

Centre for Human Genetics Bremen  
University of Bremen

**“COMPARATIVE ANALYSES OF TUMOUR RELATED GENES IN  
DOGS AS MODEL SYSTEM FOR HUMAN CANCER”**

**Dissertation**

zur Erlangung des Grades eines Doktors der Naturwissenschaften

- Dr. rer. nat. -

Dem Promotionsausschuß Dr. rer. nat.

Fachbereich Biologie/Chemie Universität Bremen

vorgelegt von

Hugo Istvan Murua Escobar

1. Gutachter: Prof. Dr. Jörn Bullerdiek

2. Gutachter: Prof. Dr. Ingo Nolte

Bremen, Mittwoch, 29. September 2004

Hiermit erkläre ich, Hugo Istvan Murua Escobar, geboren am 28.05.1973, daß für das Verfassen der vorliegenden Dissertation "Characterisation of the canine counterparts of the human tumour relevant HMGA and HMGB Protein family genes and further potential tumour relevant canine genes and evaluation as molecular targets for therapeutic approaches using the dog as model system" folgende drei Aussagen zutreffen:

1. Ich habe die Arbeit ohne unerlaubte fremde Hilfe angefertigt.
2. Ich habe keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt.
3. Ich habe die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht

Bremen, Montag, 27. September 2004

Hugo I. Murua Escobar

***"You're guaranteed to miss 100 percent of the shots you never take."***

*Wayne Gretzky*

# Contents

1	Introduction .....	8
2	Material and Methods .....	13
2.1	<i>Tissues</i> .....	13
2.2	<i>Cell Culture and Cell Lines</i> .....	13
2.3	<i>DNA Isolation</i> .....	13
2.3.1	<i>Plasmid DNA Isolation</i> .....	13
2.3.2	<i>BAC DNA Isolation</i> .....	14
2.3.3	<i>Genomic DNA Isolation</i> .....	14
2.4	<i>PCR</i> .....	14
2.5	<i>Gelelectrophoresis</i> .....	14
2.6	<i>Cloning and Sequencing of DNA Fragments</i> .....	15
2.7	<i>DNA Restriction Endonuclease Digestion</i> .....	15
2.8	<i>In Silico Analysis</i> .....	15
2.9	<i>RNA and mRNA Purification</i> .....	15
2.9.1	<i>Total RNA Purification Using TRIzol LS</i> .....	15
2.9.2	<i>Total RNA Purification Using RNeasy</i> .....	16
2.9.3	<i>mRNA Purification Using Oligotex</i> .....	16
2.10	<i>cDNA Synthesis</i> .....	16
2.10.1	<i>Synthesis Using M-MLV RT</i> .....	16
2.10.2	<i>Synthesis Using Superscript<sup>TM</sup></i> .....	17
2.11	<i>DNase Treatment</i> .....	17
2.12	<i>RNA Gelelectrophoresis and Northern Blotting</i> .....	17
2.13	<i>DNA / cDNA Probes</i> .....	17
2.14	<i>Radioactive Probe Labelling and Purification</i> .....	17
2.15	<i>Hybridisations</i> .....	18
2.15.1	<i>BAC Screening</i> .....	18
2.15.2	<i>Northern Blots</i> .....	18
3	Results .....	19
3.1	<i>Canine Gene Characterisations (Chronological Order)</i> .....	19
3.1.1	<i>The Canine HMGB1</i> .....	19
3.1.2	<i>The Canine LHCGR</i> .....	20
3.1.3	<i>The Canine HMGA1</i> .....	21
3.1.4	<i>The Canine ZNF331</i> .....	22
3.1.5	<i>The Canine CCND1</i> .....	23
3.2	<i>Canine Point Mutation Screening</i> .....	23
3.2.1	<i>Canine ras Gene Hotspot Mutation Screening</i> .....	23
3.3	<i>Gene Mapping Studies (Chronological Order)</i> .....	24
3.3.1	<i>ERBB-2</i> .....	24
3.3.2	<i>HMGA1</i> .....	24
3.3.3	<i>HMGB1</i> .....	24
3.3.4	<i>LHCGR</i> .....	24
3.3.5	<i>ZNF331</i> .....	24
3.3.6	<i>KRAS2</i> .....	24
3.3.7	<i>NRAS</i> .....	24
3.3.8	<i>AKT3</i> .....	24
3.3.9	<i>FASTK</i> .....	24
3.3.10	<i>CCND1</i> .....	24

3.4	<i>Further Publications</i> .....	25
3.4.1	<i>The HOPE-Technique</i> .....	25
3.4.2	<i>Human ZNF331 Expression in Follicular Thyroid Adenomas.</i> .....	26
3.4.3	<i>Identification of a Gene Rearranged by 2p21 Aberrations in Thyroid Adenomas.</i> .....	27
4	Discussion .....	28
5	Summary .....	38
6	References .....	40
7	Publications in Reverse Chronological Order.....	57
8	Danksagung.....	80

## Abbreviations

aa	Amino acid
AGE	Advanced glycation end products
AKT3	Protein kinase b, gamma gene
bp	Base pair
CCND1	Cyclin D1 gene
cDNA	Complementary DNA
CDS	Coding sequence
CFA	Canis familiaris
C-terminal	Carboxy terminal
Da	Dalton
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tags
FISH	Fluorescence in situ hybridisation
GAPDH	Glyceraldehyd-3-phosphat-dehydrogenase
h	Hour
ERBB-2	Human epidermal growth factor receptor 2
FASTK	FAS-activated serin/threonin kinase
HMG	High mobility group
HMGA	High mobility group protein A
HMGA1a	High mobility group protein A1 Isoform a
HMGA1b	High mobility group protein A1 Isoform b
HMGB1	High mobility group protein B1
HOPE	Hepes-glutamic acid buffer mediated Organic solvent Protection Effect
KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria Bertani
LHCGR	luteinizing hormone/choriogonadotropin receptor
M	Molar
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
min	Minute
ml	Millilitre
mM	Millimolar
M-MLV	Moloney murine leukemia virus
µl	micro litre
NaAc	Sodium acetate
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
ORF	Open reading frame
PCR	Polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RT	Reverse Transcriptase
RT-PCR	Reverse-Transcription-PCR

s	Second
SDS	Sodium dodecyl sulfat
SSC	Standard saline citrate
THADA	Thyroid adenoma associated
U	Unit
UTR	Untranslated region
V	Volt
ZNF331	Zinc finger protein 331

## Manufacturers

Ambion	Ambion, Cambridge, United Kingdom
Amersham Biosciences	Amersham Biosciences, Buckinghamshire, England
Amersham Pharmacia Biotech	Amersham Pharmacia Biotech, Freiburg, Germany
BAC/PAC Resources	BAC/PAC Resources, Okland, U.S.A.
BD Biosciences Clontech	BD Biosciences Clontech, Palo Alto, U.S.A.
Eppendorf	Eppendorf, Hamburg, Germany
Invitrogen	Invitrogen, Carlsbad, U.S.A.
DNASTAR	DNASTAR, Madison, U.S.A.
Molecular Dynamics	Molecular Dynamics, Sunnyvale, U.S.A.
Promega	Promega, Madison, U.S.A.
QIAGEN	QIAGEN, Hilden, Germany
Roche Diagnostics	Roche Diagnostics, Mannheim, Germany
Sigma Aldrich	Sigma Aldrich, Missouri, U.S.A.
Stratagene	Stratagene, California, U.S.A.

## 1 Introduction

The answer to the question which mammal would be the first to have two complete genomes of an individual sequenced seems obvious at a first glance.

***The human.***

But it is not.

***It is the dog.***

So what makes this genome so interesting for research? Where are the benefits and what is the scientific community going to earn from this double sequencing?

The canine genome offers a wide field for genetic studies on various areas like e.g. phenotypic diversity, heredity and diseases including cancer. The diversity of the different canine breeds in terms of phenotype and behaviour is unique among mammals. Up to now about 300 different canine breeds exist which compose with a wide number of mixed breeds the canine “community”. Taking into account the extreme differences in appearance and behaviour of the single breeds the identification of the genes responsible for this diversity could be significant for research e.g. in fields of development, disorders and diseases. Comparison of the mitochondrial DNA from domestic dogs and the grey wolves revealed that the difference is just 0.2% (Wayne, 1993). Considering the extreme differences in behaviour and morphology of wolves and domestic dogs the variety found in the single dog breeds suggests that the number of mutations causing these differences is small (Scott and Fuller, 1965). In fact, mitochondrial DNA comparison of different canine breeds revealed 99.9% homology (Wayne, 1993).

The different “purebred” breeding programmes carefully watched by humans including its inbreeding led to expression of various recessively inherited diseases (Patterson et al., 1988) and as side effect allowed to observe that the different breeds have particular predispositions for diseases including different types of cancer (McEwen, 1990; Nolte and Nolte, 2000). Currently, the “Canine Genetic Disease Information System” contains the clinical, pathological and genetic features of more than 370 genetic disorders (Patterson, 2000). This constitutes the largest set of naturally occurring genetic disorders in any non-human species. For those disorders for which the mode of inheritance has been defined more than 70% are inherited as

autosomal recessive, X-linked recessive, or are genetically complex, with appearance increasing with inbreeding (Ostrander et al., 2000). More than 50% of the recognised canine genetic diseases resemble the specific human genetic diseases and more than 46% of the identified genetic disorders occur predominantly in one or a few breeds (Ostrander et al., 2000).

This offers a rare opportunity for human clinical geneticists to identify breed specific disease associated genes and study them including their heredity in well documented canine pedigrees. Especially in cases where the number of human patients showing the disorder is small the high number of canine offspring is useful.

In terms of cancer the dog shows additional advantages. First of all, the dog enjoys after the human the best medical care of all organisms allowing a detailed surveillance of the cancer, its progression and therapy. Cancer studies in dogs are conducted on spontaneously developing tumours as opposed to rodent studies with tumours being experimentally induced by carcinogens or transplanted in immunocompromised animals. This suggests that the mechanisms of development in these tumours could be more similar in both species compared to rodents. Further on, dogs share the same environmental conditions with humans and are largely exposed to the same environmental agents. It is generally believed that dogs develop cancer twice as frequently as humans, and that the presentation, histology and biology of several canine cancers is similar to humans (Withrow and MacEwen 1988, 2001; MacEwen 1990). Most canine cancers progress more rapidly than their human counterparts permitting an easier observation of the tumour progression (Withrow and MacEwen, 2001). For molecular tumour analyses the canine model has the advantage of much easier accessibility of tumour samples and their corresponding “healthy” tissues due to the higher tumour incidence and lesser moral concerns compared to humans. In addition, treatment and therapy studies done in animals have the advantage that new therapeutic approaches especially single-agent trials can be done with much lower legislative restrictions. At least a dozen distinct canine cancers are hypothesized to be appropriate models for their human counterparts (Patterson, 1982; Withrow and MacEwen; 1989, MacEwen 1990; Knapp and Waters, 1997), among those osteosarcomas, mammary carcinomas, oral melanomas, lung carcinomas and malignant non-Hodgkin’s lymphomas (MacEwen, 1990).

In spite of all these advantages the development and accessibility of basic information and molecular tools to analyse the canine genome were surprisingly low at the beginning of this thesis in May 2000. The NCBI database hosted in the year 2000 about 7,000,000 EST entries of which approximately 6,000,000 were of human and rodent origin compared to just 147 canine entries (Murua Escobar, 2000). Commercially available molecular tools like multi tissue Northern Blots, cDNA libraries, BAC-/PAC libraries, micro-/macro arrays etc. were practically non existent with the exception of one constructed BAC library by Lin et al. (1999). Till 1999, of the about 215 known canine genetic diseases that showed to reassemble a counterpart in human genetic diseases only 30 were molecularly characterized and their mode of inheritance defined (for details see Patterson et al., 2000b, Switonski et al., 2004). Most of these characterisations were limited to partial or complete cDNA sequences containing the complete protein coding sequence (CDS).

The lack in gene mapping information is caused by the complicated canine karyotype showing 78 small, mostly acrocentric chromosomes that are difficult to distinguish. Till 1996 no complete standard nomenclature could be established for the dog and consent was existing just for the first 21 chromosomes according to Selden et al. (1975). Reimann et al. presented 1996 the first complete nomenclature with 460 bands which allowed to distinguish all canine chromosomes and defined their orientation. This nomenclature served as basis for all canine nomenclatures presented afterwards (Breen et al., 1999b, 1999c) and was set as standard nomenclature by the “Committee for the Standardized Karyotype of the dog” (Switonski et al., 1996). The number of laboratories capable to exactly work with Reimann’s nomenclature and reliably identify the canine chromosomes is until now (2004) very limited leading to comparatively little direct FISH mapping data. Alternatively, groups worked with linkage maps (Lingaas et al. 1997; Mellersh et al. 1997; Neff et al. 1999), canine radio hybrid (RH) maps, and by integrating both maps (Priat et al. in 1998; Mellersh et al. 2000; Breen et al., 2001; Guyon et al 2003). Currently, this map has a resolution of 900 kb and a number of 4249 markers integrated (Guyon et al. 2003; Breen et al., 2004). Chromosomal synteny studies between humans and dogs were presented in 1999 (Yang et al., 1999, 2000; Breen et al., 1999a) allowing assignment of the respective chromosomal areas. The “Gold Standard” method for reliable gene mapping remains in any case the direct FISH mapping. Existing small rearrangements, deletions and insertions in the canine

genome like those seen in the mouse genome (Wayne and Ostrander, 2004) are hardly detectable by RH mapping and thus direct FISH mapping remains the method of choice for gene localisation.

The present thesis is divided in to three main fields of the canine genome and cancer research.

Firstly, screening of canine BAC clones containing genes known to be relevant in human tumours and mapping their position in the canine genome by FISH.

Secondly, molecular cloning and characterisation of the canine counterparts of the human *HMGA* and *HMGB* Protein family genes as basis for later functional studies to be performed in a canine model system and characterisation of further potential tumour relevant canine genes.

Thirdly, screening of canine tumours for hot-spot mutations of *NRAS* and *KRAS2* genes to elucidate if known tumour mechanisms found in humans are existent in naturally occurring fibrosarcomas and melanomas of the dog as well.

HMG proteins can modify chromatin structure by bending DNA thus influencing the transcription of a number of target genes (for review Muller et al., 2001; Reeves and Beckerbauer, 2001). *HMGA1* proteins participate in regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the induction of neoplastic transformation and promotion of metastatic progression of cancer cells.

Chromosomal aberrations affecting the human *HMGA1* gene at 6p21 were described in several tumours like pulmonary chondroid hamartomas, uterine leiomyomas, follicular thyroid adenomas, and others (Williams et al., 1997; Kazmierczak et al., 1998; Tallini et al., 2000). Over-expression of the proteins of *HMGA1* is characteristic for various malignant tumours suggesting a relation between high titer of the protein and the neoplastic phenotype (Tamimi et al., 1993; Chiappetta et al., 1995, 1998; Fedele et al., 1996; Bandiera et al., 1998; Abe et al., 1999, 2000; Czyz et al., 2004). Further on, a *HMGA1* antisense strategy using an adenoviral vector treatment of induced tumours in athymic mice caused a drastic reduction in tumour size (Scala et al., 2000).

Beside its function as an architectural transcription factor, *HMGB1* can also be secreted by certain cells, e.g. macrophages (Wang et al., 1999). As an extracellular protein *HMGB1* is a ligand for the receptor for advanced glycation end products (RAGE) (Hori et al., 1995; Parkkinen and Rauvala, 1991; Parkkinen et al., 1993) thus

activating key cell signalling pathways, such as p38<sup>MAPK</sup>, JNK, and p42/p44<sup>MAPK</sup> and playing an important role in inflammation and tumour metastasis (Taguchi et al., 2000; Liotta and Clair, 2000). HMGB1 is able to bind to cisplatin-DNA-adducts (Pil and Lippard 1992) and sensitises cancer cells to cisplatin by shielding its major DNA adducts from nucleotide excision repair (He et al. 2000). He et al. (2000) have shown that in oestrogen receptor positive human breast cancer cells oestrogen can significantly increase the effect of cisplatin by causing an overexpression of *HMGB1*. This finding has led to the conclusion that oestrogen treatment prior to cisplatin therapy may sensitise the cancer cells against that drug.

Point mutations of the so-called hot-spot codons 12, 13, and 61 affecting genes of the *ras* family are assumed to be among the most important alterations in human tumourigenesis (Hahn et al., 1994; Arber et al., 1999). These *ras* proteins play an important role as signal transmitters which are activated by the binding of growth factors initiating cell division. Mutations in *ras* genes are assumed to remove the time limit of the cell stimulating signals which results in uncontrolled cell division by affecting their autotermination process (Sigal et al., 1988, 1988a; Singer 1992; Park 1995; Watzinger et al., 1998). Just one report of a *ras* mutation screening including canine fibrosarcoma samples has been published (Watzinger et al., 2001) and also studies about *KRAS2* mutations in canine melanomas are completely missing. In canine melanomas so far almost no hot-spot *NRAS* mutations were described with one exception: recently Mayr et al. (2003b) found 2 of 16 melanomas to be affected by mutations in codon 61.

To achieve the primary goals of this thesis the following work was conducted:

Firstly, the position of ten canine genes was localised by direct canine gene FISH mapping.

Secondly, the canine *HMGA1*, *HMGB1*, *CCND1*, *ZNF331* genes were molecularly characterized.

Thirdly, thirteen canine fibrosarcomas, two feline fibrosarcomas and eleven canine melanomas were screened for *ras* gene point mutations, particularly within the mutational hot-spot codons.

Additionally, it was participated in studies involving the human *ZNF331* and *THADA* genes, and in the evaluation of the new fixative HOPE for molecular methods.

## **2 Material and Methods**

### **2.1 Tissues**

The tissues used were provided by the Small Animal Clinic, School of Veterinary Medicine, Hanover, Germany. Samples were taken during surgical treatment or straightly after the animal was “put to sleep”. Tissues were put into cryo-tubes, labelled and immediately shock-frozen in liquid nitrogen. The tubes were sorted and stored at -80°C

The breeds represented were Alsatian, Bull Terrier, Collie, Dachshund, Doberman Pinscher, German Shorthaired Pointer, Golden Retriever, Jack Russell Terrier, Kangal, Munsterland, West Highland Terrier, Yorkshire Terrier and crossbreeds.

### **2.2 Cell Culture and Cell Lines**

The cell lines used were provided by the Centre for Human Genetics, University of Bremen, Bremen, Germany.

Cell line ZMTH3 was derived from a canine pleomorphic adenoma.

Cell line MTH52C was derived from malignant small cell tumour.

Cell line MTH53A was derived from healthy canine mammary tissue.

### **2.3 DNA Isolation**

#### *2.3.1 Plasmid DNA Isolation*

Plasmid DNA preparations were done using the “QIAprep Spin Miniprep Kit” (Qiagen) following the manufacturers instructions (QIAprep Miniprep Handbook, Page 22-23, QIAprep Spin Miniprep Kit Protocol). 5 ml LB overnight cultures were used for standard procedure DNA isolation. The DNA was eluted in EB buffer and the concentrations measured photometrically.

### **2.3.2 BAC DNA Isolation**

BAC DNA preparations were done using the “QIAGEN Plasmid Maxi Kit” (Qiagen) following the manufacturers instructions (QIAGEN Plasmid Purification Handbook Page 47- 51, Protocol “Very Low-Copy Plasmid/Cosmid Purification”). 5 ml LB overday (8 h) cultures were used as starter cultures for inoculation of 250 ml overnight cultures for standard procedure BAC DNA isolation. The BAC DNA was eluted in EB buffer and the concentration measured photometrically.

### **2.3.3 Genomic DNA Isolation**

Genomic DNA isolations from blood, tissue and cell culture were done using the “QIAamp DNA Mini Kit” (Qiagen) following the manufacturers instructions (QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook). DNA from blood was obtained using 200µL EDTA blood (Page 27-29, Blood and Body Fluid Spin Protocol). For DNA tissue isolation 25 mg fresh or frozen tissue were used (Page 33 - 36, Tissue Protocol). Isolations from cell culture were done using  $5 \times 10^6$  cells (Page 49, Protocol for cultured cells). The DNA was eluted in EB buffer and the concentration measured photometrically.

## **2.4 PCR**

DNA fragment amplification with PCR was done using gene specific primers following the standard (Invitrogen) protocol (Basic PCR Protocol). The annealing temperatures and minor modifications were varied according to the used primer (for details see the respective publications). The PCR reaction itself was done in an Eppendorf Mastercycler Gradient (Eppendorf) in 0,2 ml Eppendorf PCR reaction tubes. PCR product purification was done using “QIAquick PCR Purification Kit” (Qiagen) following the “QIAquick Spin Handbook, Page 18, QIAquick PCR Purification Kit Protocol”.

## **2.5 Gelelectrophoresis**

The electrophoretic separation of the DNA molecules was done depending on the DNA fragment size in 0,7 – 4,0 % agarose gels. The samples were mixed with an adequate volume of 6x loading dye and separated at 6 V/cm for 1 – 24 h.

Visualisation of the DNA was done using Ethidiumbromide and UV light at 254 nm.

## **2.6 Cloning and Sequencing of DNA Fragments**

PCR DNA fragments to be cloned were recovered with QIAEX II (Qiagen) following the manufacturers instructions (QIAEX II Handbook, Page 12-13, QIAEX II Agarose Gel Extraction Protocol). The fragments were cloned in pGEM-T Easy Vector System (Promega) following the manufacturers instructions using 50ng vector DNA and 1  $\mu$ l PCR product (pGEM-T and pGEM-T Easy Vector Systems Technical Manual No. 042, Page 7 ,Protocol for Ligations Using the pGEM-T and pGEM-T Easy Vectors and the 2X Rapid Ligation Buffer).

## **2.7 DNA Restriction Endonuclease Digestion**

DNA endonuclease digestions were done using various restriction enzymes and their specific buffers and temperatures following the manufacturers instructions. DNA quantities over 5  $\mu$ g DNA were digested with 20 U enzyme overnight. DNA quantities minor then 5  $\mu$ g were digested applying 5 U enzyme for 3 – 5 h.

## **2.8 In Silico Analysis**

The genomic DNA contigs, cDNA contigs, protein predictions, and the homology alignments were created with Lasergene software (DNASStar).

## **2.9 RNA and mRNA Purification**

### **2.9.1 Total RNA Purification Using TRIzol LS**

Total RNA was isolated from canine tissue and cell culture using TRIzol LS (Invitrogen). For cell culture total RNA isolation the cell culture medium was removed from the tissue culture flask, 1 ml TRIzol LS added and the cells resuspended by pipetting for homogenisation.

The tissue samples were reduced to small pieces in a cell culture dish in 1 ml TRIzol LS for homogenisation. All following steps were performed following the manufacturers protocol (TRIzol LS Reagent Protocol). Finally the RNA pellets were washed twice in 70% ethanol, dried at room temperature and resuspended in A.

bidest. Before storing at  $-80^{\circ}\text{C}$  all sample concentrations were measured photometrically.

### *2.9.2 Total RNA Purification Using RNeasy*

Total RNA was isolated from canine tissue and cell culture using “QIAGEN RNeasy Mini Kit” (Qiagen) following the instructions of the “RNeasy Mini Handbook”. For cell culture total RNA isolation the cell culture medium was removed from the tissue culture flask, and the cells resuspended in the required amount of RLT buffer. All following steps were performed following the manufacturers protocol (RNeasy Mini Protocol for Isolation of Total RNA from Animal Cells, Page 31-35). The tissue samples were homogenised using a disperser tool (Ultra-Turrax) in the required amount of RLT buffer. All following steps were performed following the manufacturers protocol (RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues, Page 50-55). Before storing at  $-80^{\circ}\text{C}$  all sample concentrations were measured photometrically.

### *2.9.3 mRNA Purification Using Oligotex*

mRNA purification from total RNA was done using the “Qiagen Qligotex mRNA Mini Kit” (Qiagen) following the manufacturers instructions (Oligotex Handbook, Page 20 – 22, Oligotex mRNA Spin-Column Protocol). The purifications were started with up to 1 mg total RNA. The mRNA was eluted with QEB buffer, measured photometrically and stored at  $-80^{\circ}\text{C}$ .

## **2.10 cDNA Synthesis**

### *2.10.1 Synthesis Using M-MLV RT*

cDNA was synthesised from total RNA and mRNA using 3'-RACE adaptor primer AP2 (AAGGATCCGTCGACATC(17)T), 5  $\mu\text{g}$  total RNA or 500 ng mRNA, and 200 U M-MLV RT reverse transcriptase according to the manufacturer's (Invitrogen) instructions (M-MLV Reverse Transcriptase Protocol, review 091002).

### **2.10.2 Synthesis Using Superscript™**

cDNA was synthesised from total RNA and mRNA using 3'-RACE adaptor primer AP2 (AAGGATCCGTCGACATC(17)T), 5 µg total RNA or 500 ng mRNA, and 200 U Superscript™ RT Reverse Transcriptase according to the manufacturer's (Invitrogen) instructions (Superscript™ Reverse Transcriptase, Protocol review 260903).

### **2.11 DNase Treatment**

To avoid genomic DNA contamination in the RNA samples a DNase digest of each RNA sample was performed using DNA-free according to the manufacturer's (Ambion) instructions using 2 U DNase I / 10µg RNA (Protocol "DNase Treatment and Removal Reagents", Version 0204).

### **2.12 RNA Gelelectrophoresis and Northern Blotting**

RNA gelelectrophoresis and Northern blotting was performed following the instructions of the Stratagene (Stratagene) "Northern Transfer Protocol" (Messenger RNA Isolation Kit, Instruction Manual, Page 13 – 15).

### **2.13 DNA / cDNA Probes**

Canine cDNA and genomic DNA probes were used for hybridisation of canine Northern Blots and canine BAC Screening, respectively. The probes were generated by PCR using gene specific primer on cDNA or genomic DNA of various canine tissues, blood or cell cultures. The obtained PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM-T Easy Vector System (Promega) and sequenced for verification.

### **2.14 Radioactive Probe Labelling and Purification**

Probe labelling was performed either using the "Megaprime™ DNA Labelling System" (Amersham Pharmacia Biotech) or "Random Primed DNA Labeling Kit" (Roche Diagnostics) following the manufacturers protocols with 50 µCi( $\alpha^{32}\text{P}$ )dCTP and 50 ng probe DNA for Northern and Southern Blots or 250 µCi( $\alpha^{32}\text{P}$ )dCTP and 250 ng probe DNA for BAC Filter Screening. The ( $\alpha^{32}\text{P}$ )dCTP radioactive nucleotides

were synthesised by Amersham Pharmacia Biotech. Purification of the labelled probe was performed using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) following the manufacturer's instructions.

## **2.15 Hybridisations**

### *2.15.1 BAC Screening*

Canine genomic DNA probes were used for hybridisation of canine RPCI-81 BAC/PAC filter (BACPAC Resources). The probes were generated by PCR using gene specific primer on genomic DNA of the canine tissue, blood or cell culture. The obtained PCR product was separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM-T Easy Vector System (Promega) and sequenced for verification. The probe labelling was performed as described in the manufacturer's protocol with 250 ng DNA and 250  $\mu\text{Ci}(\alpha^{32}\text{P})\text{dCTP}$ . Purification of the labelled probe was done using Sephadex G-50 Nick Columns. Hybridisation and analysis were performed according to manufacturer's protocol (Hybridisation of High Density Filters). Signals were visualised using a STORM phosphorimager (Molecular Dynamics).

### *2.15.2 Northern Blots*

Northern Blot hybridisations were done using the PERFECTHYB PLUS hybridisation solution (Sigma-Aldrich). Prehybridisation was carried out for 30 min and hybridisation for 2.5 h at 68°C. The membrane was washed for 5 min at room temperature in 2 x SSC / 0.1% SDS, and twice for 20 min at 68°C in 0.5 x SSC / 0.1% SDS. Signals were visualised using a STORM phosphorimager (Molecular Dynamics).

### 3 Results

#### 3.1 Canine Gene Characterisations (Chronological Order)

To use the dog as model system for human diseases in basic research, cancer research and drug discovery the knowledge and characterisation of the corresponding canine genes is precondition. In this study gene characterisation work was done for five canine genes with focus on the canine *HMG* genes. The results are presented in chronological order.

##### 3.1.1 The Canine *HMGB1*

XIII: Murua Escobar H et al., Cytogenetics and Genome Research, 101(1): 2003

VIII: Meyer B et al., Anticancer Research, 24(2B): 2004

The complete canine *HMGB1* cDNA consists of 2236 bp encoded by five exons similar to the human transcript (GenBank acc. no. AY135519). The identity of the complete molecule compared to its human counterpart is 90.8% whereas the ORF is 95.4%. The derived canine protein consists of 215 aa with a molecular weight of 24892.67 Da. Comparison to the human protein showed 100% homology of the canine counterpart. Northern Blot analyses were performed to define a basic expression pattern in canine heart, lung, muscle, kidney, and spleen tissue. Except for the kidney tissue that showed no detectable signal, all samples revealed two transcripts similar to the signals obtained in human fibroblasts of about 1.4 and 2.4 kb. Northern Blot hybridisation on a series of 5 osteosarcomas, one fibrosarcoma and one leiomyosarcoma sample resulted in the detection of the two *HMGB1* transcripts. In order to quantify the expression of *HMGB1*, the blot was rehybridised with a canine *GAPDH* specific cDNA probe. After summarising the intensities of the 1.4 and 2.4 kb *HMGB1* signals, the *HMGB1*-RNA / *GAPDH*-RNA ratios were calculated. Values obtained by Northern Blot analysis for the osteosarcoma samples varied between 0.52 and 1.31, while the fibrosarcoma and the leiomyosarcoma showed ratios of 0.73 and 0.24, respectively. Semi-quantitative duplex RT-PCR suitable for detecting intertumoural variation of *HMGB1* expression in relation to expression of the house-keeping gene *GAPDH* showed values for the osteosarcoma samples which varied

between 0.72 and 1.28, while the ratios for the fibrosarcoma and the leiomyosarcoma were 0.73 and 0.42, respectively. In order to determine the comparability of the results obtained by the Northern Blot hybridisation and RT-PCR analyses, mean values for each test series were calculated, set to one, and relative expression levels were determined. Statistical analysis using the Pearson's Correlation Test revealed a significant correlation between the relative *HMGB1* expression level obtained by Northern Blot hybridisation and the level obtained by the established RT-PCR ( $r=0.8919$ ,  $p=0.0071$ ). At the genomic level the canine *HMGB1* gene exon/intron structure is similar to the human ortholog consisting of five exons and four introns. While the homologies of the exons, lying between 89.4% and 98.7%, are extremely high the homologies of the amplified introns 2 – 4 vary between 20.3% – 59.0%. Approaches to amplify the canine intron 1 resulted in unspecific PCR products.

### *3.1.2 The Canine LHCGR*

XI: Santos SE et al., *Animal Genetics*, 35(1): 2004

Part of exon 11 of the canine luteinizing hormone receptor (*LHCGR*) gene was PCR amplified (960 bp) and sequenced. The canine exon 11 fragment shared 89%, 89%, 92% and 88% identity to the human, bovine, porcine and murine *LHCGR* sequences respectively. Two polymorphic sites were identified in codons encoding amino acid positions Ile 378 and Val 397 of *LHCGR*.

### 3.1.3 The Canine *HMGA1*

X: Murua Escobar H et al., Gene, 14(330): 2004

The characterisation of the canine *HMGA1* cDNAs revealed that the complete canine *HMGA1* cDNA spans six exons and codes for two splicing variants *HMGA1a* with 1836 bp and *HMGA1b* with 1803 bp. The splicing variants showed the “typical” 33 bp gap difference which is conserved across various species. The homology of the canine cDNAs to their human counterparts is 80.6% for both splicing variants. The 5'-UTR, CDS, and the 3'-UTR showed homologies of 95.6, 95.1 and 74.7%, respectively. The canine *HMGA1a* protein is a 107 amino acid molecule with a calculated weight of 11,674.97 Da and *HMGA1b* a 96 amino acid molecule with a calculated weight of 10,677.85 Da. For twelve different canine breeds the splicing variants *HMGA1a* and *HMGA1b* were characterised. The comparison of the protein coding sequences for these twelve canine breeds revealed one amino acid change in a single breed. Sample 2 (Teckel) showed in its *HMGA1b* transcript a nucleotide transition from A to G at the first base of codon 64 leading to an aa replacement from threonine to alanine and a new restriction recognition site for *AluI* causing four (58, 100, 158, 176 bp) instead of three fragments (58, 100, 334 bp) to appear in an *AluI* digest. The substitution was missing in the corresponding *HMGA1a* transcript of the dog suggesting a heterozygous genotype. Northern Blot analysis containing total RNA from canine spleen, heart, lung, muscle, and kidney tissue samples showed, with exception of the kidney total RNA and one of two heart samples (Trizol method) a weak signal of approximately 1.8 kb for all total RNA samples, while the poly A RNA spleen sample revealed a distinct signal. Rehybridisation with a canine *GAPDH* probe showed signals corresponding to approximately 1.3 kb in all but the Trizol method, indicating a degradation of the Trizol-prepared RNA.

### 3.1.4 The Canine ZNF331

VII: Meiboom M et al., *Animal Genetics*, 35(3): 2004

The composed canine *ZNF331* cDNA (GenBank acc. no. AY375188) consists of 2148 bp including the full ORF. Comparison of canine and human *ZNF331* showed high homology on the nucleotide level with sequence identities of 87.3% in the KRAB-A box and 87.2% in the zinc finger domain. The spacer region showed 77.3% homology to its counterpart. Overall, the nucleotide sequence identity of canine and human *ZNF331* is 85.3% in the ORF and the adjacent 50 bp of the 5' UTR containing the ATG start codon. The 3' UTRs and remainings of the 5' UTRs showed little sequence identity with 35.6% and 39.8%, respectively.

At the amino acid sequence level, the canine and the human *ZNF331* revealed a 92.9% homology for the KRAB-A boxes and 96% for the zinc finger domains. The spacer regions revealed only 59.6% homology in the amino acid sequence. The zinc finger domain of canine *ZNF331* is extended by 26 amino acids resulting from a nucleotide deletion, when compared to human cDNA sequence, directly upstream of the TGA stop codon leading to a shift in the open reading frame. Based on the sequence data obtained, it can be deduced that canine *ZNF331* is composed of a 1813 bp 3' exon containing the 3' UTR, the zinc finger domain and the spacer, a 126 bp exon containing the KRAB-A box, and a so far unknown number of 5' exons comprising 209 bp.

Expression studies using Northern Blots containing mRNA from various canine tissues including testis and a canine *ZNF331* spacer-specific probe did not reveal transcripts of canine *ZNF331* which points to a very low expression level of this gene.

### 3.1.5 The Canine *CCND1*

II: Meyer B et al., *Animal Genetics*, 35(5): 2004

For characterisation of the canine *CCND1* gene and the corresponding protein a cDNA from a canine osteosarcoma was screened with primers specific for the ORF of human cyclin D1. *In silico* analyses allowed the composition of a 1246 bp cDNA contig (GenBank acc. no. AY620434), showing 90.4% sequence identity of the canine ORF, 68.5% in the 3' UTR, and 74.7% 5' UTR respectively, compared to the human counterpart (NM\_053056). In accordance with the human orthologue the deduced canine protein comprises 295 aa with 93.3% homology between the two species.

## 3.2 Canine Point Mutation Screening

### 3.2.1 Canine *ras* Gene Hotspot Mutation Screening

I: Murua Escobar H et al., *Aniticancer Research* (in press)

To elucidate if *ras* mutations exist in these naturally occurring tumours in dogs, thirteen canine fibrosarcomas, two feline fibrosarcomas and eleven canine melanomas were screened for point mutations, particularly within the mutational hotspots. Four of the analysed twenty-six samples showed nucleotide exchanges in the screened canine exons. None of the found exchanges affected the *ras* hotspot codons 12, 13 and 61. One fibrosarcoma sample (Berger de Brie) showed three changes affecting *KRAS2* exon 1 codon 23 (CTA→TTA, no amino acid exchange), exon 2 codon 53 (TTG→TAG, Leu→stop codon), and *NRAS* exon 1 codon 10 (GGA→GAA, Gly→Glu). Two other fibrosarcomas (Kuvasz and Poodle) each showed one nucleotide exchange in *KRAS2* exon 2 affecting codon 48 (GGA→GAA, Gly→Glu) and codon 70 (CAG→CTG, Gln→Leu), respectively. *NRAS* exon 1 codon 22 (CAG→CTG, Gln→Leu) was affected in a melanoma sample (crossbreed). The screening of *NRAS* exon 2 revealed no nucleotide exchanges among the canine sequences. The described nucleotide differences between the canine and feline sequences in *NRAS* exon 2 and *KRAS2* exon 2 were detected.

### 3.3 Gene Mapping Studies (Chronological Order)

(Publications see Table I)

Direct canine gene mapping data is very rare due to the complicated canine karyotype and the controversially discussed nomenclature. Since Reimann et al. (1996) established a complete nomenclature which allowed to identify and distinguish all canine chromosomes direct gene mapping by FISH has been carried out by a very few different groups who are capable to identify the canine chromosomes correctly. A total of 10 canine genes (Table I) could be localized during this thesis.

Table I

Gene	Localisation on CFA	Publication (in chronological order)
3.3.1 <i>ERBB-2</i>	1q13.1	<i>XVI: Murua Escobar et al., 2001, Cytogenetics Cell Genetics, 94(3-4)</i>
3.3.2 <i>HMGA1</i>	23	<i>XV: Becker et al., 2003, Animal Genetics, 34(1)</i>
3.3.3 <i>HMGB1</i>	25	<i>XIII: Murua Escobar et al., 2003, Cytogenetics and Genome Research, 101(1)</i>
3.3.4 <i>LHCGR</i>	10	<i>XI: Santos et al., 2004, Animal Genetics, 35(1)</i>
3.3.5 <i>ZNF331</i>	1	<i>VII: Meiboom et al., 2004, Animal Genetics, 35(3)</i>
3.3.6 <i>KRAS2</i>	22	<i>VI: Winkler et al., 2004, Animal Genetics, 35(4)</i>
3.3.7 <i>NRAS</i>	17	<i>V: Richter et al., 2004, Animal Genetics, 35(4)</i>
3.3.8 <i>AKT3</i>	7	<i>IV: Murua Escobar et al., 2004, Animal Genetics, 35(4)</i>
3.3.9 <i>FASTK</i>	16	<i>III: Meyer et al., (submitted), Animal Genetics</i>
3.3.10 <i>CCND1</i>	17	<i>II: Meyer et al., 2004, Animal Genetics, 35(5)</i>

### 3.4 Further Publications

#### 3.4.1 The HOPE-Technique

IX. Goldmann et al., Pathology-Research and Practice, 200: 2004

With increasing application possibilities for conventional histological and molecular diagnostics the demand for a “multi-application-conservation-method” is increasing. For formalin-fixed, paraffin embedded (FFPE) tissues, the latter application possibilities are very limited. The HOPE-(Hepes-glutamic acid buffer mediated **O**rganic solvent **P**rotection **E**ffect) technique comprises a new protection-solution allowing all pathologic routine investigations.

It was analysed whether RNA isolated from HOPE fixed tissue was suitable for Northern Blot and microarray application.

Qualitative integrity test by capillary electrophoresis and spectrometer revealed that the two RNA samples used for array hybridization showed two prominent peaks within the elution profile, corresponding to 28S and 18S rRNA. The ratio of 28S to 18S rRNA peak areas was >1.5, confirming the integrity of the RNA. The sizes of the amplified RNAs ranged from 0.2 to 3.0 kb, with an average length of 1.0 kb.

Cy3 and Cy5 fluorescently labeled cDNAs were combined and simultaneously hybridized to a predefined microarray, comprising 642 oncologically relevant genes, to determine whether or not this RNA is suitable for array experiments. It turned out that RNA prepared from HOPE-fixed tissue can be successfully used for microarray hybridization. We found that 46 of 642 genes were >2-fold upregulated, and that 45 of 642 genes were >2-fold downregulated when hybridization results of grade 3 versus grade 2 samples were compared with each other.

Northern Blot analyses using total RNA isolated from seven HOPE-fixed breast cancer samples showed a *GAPDH*-specific transcript of 1.3 kb and two splicing variants of 1.4 and 2.4 kb of the high mobility group protein gene B1 (*HMGB1*).

### 3.4.2 Human ZNF331 Expression in Follicular Thyroid Adenomas.

XII Meiboom et al., Cytogenetics and Genome Resesearch, 101(2): 2003

Sequencing of 5'RACE products revealed seven new splicing variants of *ZNF331*. In each investigated clone the exons 5, 4, and 3 representing the zinc finger region, the KRAB domain and the part of 5' UTR including the ATG start codon were identical. Differences in cDNA sequence were upstream of exon 2 except for two clones (*ZNF331b* and *ZNF331f*), which had an additional 30 bp insertion between exon 2 and 3. Clone *ZNF331c* revealed an extended exon 2, clones *ZNF331d, e, f, and g* contained a 67 bp exon upstream of exon 2, which differs in only 4 bp from exon 2. Clones *ZNF331d, e, f, and g* containing the duplicated exon 2 revealed sequence homology with ESTs from the NCBI database. All sequences correspond to the known genomic sequence.

Alignment of cDNA sequences with genomic DNA allowed the prediction of three transcription start sites for the *ZNF331* splicing variants. The transcription start sites for *ZNF331* and *ZNF331a, b, and c* are supposed to be upstream of exon 1 indicating that these splicing variants span about 23 kbp on the genomic level. Further transcription start sites are most likely about 16 kbp (*ZNF331d, e*) and 32 kbp (*ZNF331f, g*) upstream of the transcription start point for *ZNF331* and *ZNF331a, b, and c*. Taking this into account, at the genomic level *ZNF331* spans approximately 56 kbp.

Northern Blot hybridisation with different specific *ZNF331* probes on poly(A)<sup>+</sup> RNA of several normal tissues (human MTN IV membrane, Clontech), and thyroid carcinoma cell lines TPC1, NIM1, FRO, and ARO, and human fibroblasts revealed various transcripts ranging from 2.1 - 6.2 kbp. Unique expression of a 3.4 kbp transcript was seen in cell lines of thyroid tumours with structural alterations in chromosome 19q13.4.

### 3.4.3 Identification of a Gene Rearranged by 2p21 Aberrations in Thyroid Adenomas.

XIV: Rippe et al., Oncogene, 22(38): 2004

In two human thyroid adenoma cell lines with aberrations in 2p21, a gene spanning the chromosomal breakpoint was identified and named *THADA*. *THADA* cDNA contains 38 exons with at least one alternative splice variant coding for a deduced 1663 aa protein with a predicted molecular weight of 187 kDa. Multiple Northern Blot analysis detected a 6.2 kbp transcript in all investigated tissues. RACE-PCR experiments done in the cell lines S325/TSV40 and S533/TSV40 resulted in detection of *THADA* transcripts diverging from *THADA* after exon 28. Database search revealed that the sequences fused to *THADA* in S325/TSV40 map to the chromosomal band 3p25 and in S533/TSV40 to chromosomal band 7p15. In order to confirm the fusions in both cell lines, RT-PCR analyses were performed. Amplification products corresponding to those found by RACE-PCR were obtained for both cell lines, thus confirming the *THADA-FUS3p* and *THADA-FUS7p* fusions. In both cell lines, *THADA* stops after exon 28 followed by the fused sequences from either of the translocation partners.

## 4 Discussion

As witnessed by a number of recent articles (Kuska, 1999; Kingman et al., 2000; Ostrander et al., 2000; Vail and MacEwen, 2000) a growing number of scientists predict that human genetics will be “going to the dog” in this century (Kuska, 1999). Due to the emerging advantages of numerous canine diseases as a genetic model for human orthologues, the dog could join the mouse as the species of choice to unravel genetic mechanisms e.g. of cancer predisposition, development, and progression. As reviewed by Patterson (2000b) and Switonski et al. (2004) for 30 canine inherited diseases, most of them showing human counterparts, the molecular gene characterisation allowed to identify the mutations causing the disorders. For these 30 genes at least 60% showed similar molecular background to their human counterpart causing the disease and similar clinical symptoms (Switonski et al., 2004).

The aim of this thesis was to characterise the canine counterparts of the human HMGA and HMGB protein family genes and characterisation of further potential tumour relevant canine genes also including analyses of their mutations. Knowledge of the gene structures would be the precondition to evaluate the canine gene products as potential targets for therapeutic approaches using the dog as model system for human disorders and for development of new therapies for both species.

The human *HMG* genes are reported to be involved in a wide range of various cancers being re-expressed, over-expressed or even acting as extracellular ligands (Reeves and Beckebauer, 2001, Muller et al., 2001).

The characterisation of the canine *HMGA1* cDNAs revealed that the cDNAs coding for the two canine splicing variants *HMGA1a* and *HMGA1b* are similar to the human transcripts (GenBank acc. nos. AY366390 and AY366392). The splicing variants showed the “typical” 33 bp gap difference which is conserved across various species such as human, mouse, hamster, and rat (GenBank acc. nos. BC013455, NM\_016660, A7193763, NM\_139327, A7511040). The identity of the canine cDNAs to their human counterparts is 80.6% for both splicing variants. The 5'-UTR, CDS, and the 3'-UTR showed homologies of 95.6%, 95.1%, and 74.7%, respectively.

Homologies of the canine CDS with the CDS from mouse, hamster and rat on nucleotide level vary from 90.4% to 93.1%.

The canine HMGA1a and HMGA1b protein sequences were deduced from the respective cDNA sequences. Homology comparison to the human counterparts (GenBank acc. nos. P17096, X14957) showed 100% homology within the three “AT-hooks” and the acidic carboxy-terminal domain.

Comparison of the canine and human HMGA1a and HMGA1b proteins with the described mouse, rat and hamster molecules showed aa changes in positions 5, 34, 69, 75, and 78 of HMGA1a and positions 5, 34, 58, 64, and 67 of HMGA1b, respectively (Johnson et al., 1988; Johnson et al., 1989; Friedmann et al., 1993; Aldrich et al., 1999; Sgarra et al., 2002; Strausberg et al., 2002; Sgarra et al., 2003).

According to the definition of the AT-hooks (HMGA1a: I aa 21 – 31, II aa 53 – 63, III aa 78 – 89; HMGA1b: I aa 21 – 31, II aa 42 – 52, III aa 67 – 78) by Reeves and Nissen (1990) and Reeves (2000), none but the aa exchange at position 78 (HMGA1a) or 67 (HMGA1b), respectively, do affect the AT-hooks in either species. The exchange at position 78 leads to a difference in the third AT-hook of mouse and hamster when compared to the other species. According to the definition of the AT-hooks (HMGA1a: I aa 23 – 31, II aa 55 – 70, III aa 81 – 89; HMGA1b: I aa 23– 31, II aa 44– 59, III aa 70 – 78) by Huth et al. (1997), this aa exchange does not affect the third AT-hook. Following this definition, the second AT-hook is affected by the aa exchange at position 69 (HMGA1a) or 58 (HMGA1b), respectively.

Due to the identical structure of the canine HMGA proteins to the respective human molecules, therapeutic approaches applied in dogs could be a better suited model for the development of human therapies than approaches tested in other organisms.

For twelve different canine breeds the splicing variants *HMGA1a* and *HMGA1b* were compared. The comparison of the characterised protein coding sequences for these twelve canine breeds revealed one amino acid change in a single breed (Teckel, HMGA1b transcript). The nucleotide substitution was missing in the corresponding HMGA1a transcript of the dog suggesting a heterozygous genotype. A possible PCR artefact seems rather unlikely since the nucleotide transition was verified as described in the corresponding publication (Murua Escobar et al., 2004a). Nucleotide exchanges causing no amino acid substitution were not taken into account for further analyses.

Expression of human *HMGA1* is detectable at very low levels or is even absent in adult tissues whereas it is abundantly expressed in embryonic cells (Chiappetta et al., 1996). According to the expression patterns seen in humans the canine Northern Blot analyses in adult tissues revealed low expression in spleen, heart, lung, and muscle tissue. In humans the *HMGA1* gene locus 6p21 is often affected by aberrations leading to an up-regulation of the HMGA1 protein in benign mesenchymal tumours, e.g. lipomas, uterine leiomyomas, pulmonary chondroid hamartomas, and endometrial polyps (Williams et al., 1997; Kazmierczak et al., 1998; Tallini et al., 2000). Transcriptional activation due to a chromosomal alteration of *HMGA1* is probably an early and often even primary event of cancer development. In contrast, in dogs the gene locus of the canine *HMGA1* gene does not map to a region frequently affected by chromosomal rearrangements (Becker et al., 2003). Therefore, in contrast to humans the activation of that gene as a result of chromosomal translocations does not seem to play a considerable role in canine tumours.

*HMGA1* expression in human malignant epithelial tumours seems to be associated with an aggressive behaviour of the tumours. Over-expression of *HMGA1* was reported for a number of malignancies including thyroid, prostatic, pancreatic, uterine cervical, and colorectal cancer (Tamimi et al., 1993; Chiappetta et al., 1995; Fedele et al., 1996; Bandiera et al., 1998; Chiappetta et al., 1998; Abe et al., 1999; Abe et al., 2000; Czyz et al., 2004; Takaha et al., 2004). The correlation between *HMGA* expression and tumour aggressiveness in some of these malignancies has led to the conclusion that *HMGA* expression may present a powerful diagnostic and prognostic molecular marker.

The causal role of *HMGA1* expression in the progression of carcinomas has been elucidated by a set of *in vitro* experiments involving *HMGA1* sense and antisense transfection assays (Wood et al., 2000a; Wood et al., 2000b; Reeves et al., 2001). A proof of concept for a therapy aimed at the down-regulation of HMGA protein in tumours has been presented by Scala et al. (2000) who were able to show that an *HMGA1* antisense strategy using an adenoviral vector treatment of tumour cell xenograft athymic mice caused a drastic reduction in tumour size.

So far no studies analysing the *HMGA1* expression patterns in canine tumours have been carried out. Since these tumours occur spontaneously in dogs as well as in humans a canine *in vivo* model system could have significant value for research and

drug development. Due to the similarities of human and canine tumours the transfer of such experimental approaches could benefit cancer research in either species.

The homology of the complete canine *HMGB1* cDNA to its human counterpart (GenBank acc. no. AY135519) is 90.8% whereas the ORF is 95.4%, 5' UTR 95.6%, and the 3' UTR 88.6% respectively. Comparison of the deduced canine protein to the human molecule showed 100% homology. Differences between the canine HMGB1 and the mouse and rat protein are two aa changes in the acidic carboxy-terminal domain at positions 189 and 202 (Paonessa et al., 1987; Ferrari et al., 1994). The bovine molecule (GenBank acc. no. P10103) shows one aa change at position 207 compared to its human and canine counterparts.

Northern Blot analysis showed *HMGB1* expression in canine heart, lung, muscle, kidney, and spleen tissue. Except for the kidney tissue that showed no detectable signal, all samples revealed two transcripts similar to the signals of about 1.4 and 2.4 kb obtained in human fibroblasts. Human *HMGB1* transcripts of this size had been detected in multiple normal tissues (Rogalla et al., 1999) and several breast cancer samples (Flohr et al., 2001). Interestingly, *HMGB1* gene expression can be induced by oestrogens in breast cancer MCF-7 cells probably due to an upregulation of the gene that thus can be considered oestrogen-responsive (Chau et al., 1998). Additionally, it has been shown that HMGB1 is able to bind to cisplatin-DNA-adducts (Pil and Lippard, 1992) and sensitises cancer cells to cisplatin by shielding its major DNA adducts from nucleotide excision repair (He et al., 2000). He et al. (2000) have shown that in oestrogen receptor positive human breast cancer cells oestrogen can significantly increase the effect of cisplatin by causing an overexpression of *HMGB1*. This finding has led to the conclusion that oestrogen treatment prior to cisplatin therapy may sensitise the cancer cells against that drug. Accordingly, a clinical trial for the treatment of gynaecological tumours with cisplatin has already been approved by the Food and Drug Administration (He et al., 2000). The quantitation of the intratumoural *HMGB1* expression level may be of high impact for a cisplatin/carboplatin therapy for two reasons. Firstly, it may predict the clinical outcome of the therapy; secondly, it may influence the therapy protocol as, for example, tumours showing a high *HMGB1* expression level may be treated with a lower amount of this antitumour drug. Our studies in dogs detected strong intertumoural variation of *HMGB1* expression by Northern Blot analysis and by semi-

quantitative RT-PCR in seven canine sarcomas (Meyer et al., 2004). Taking into account the previously described facts the quantitation of the *HMGB1* expression level in canine sarcomas could become of great value for future design of therapy protocols as up to date no features have been identified allowing clinicians to predict the response to cisplatin or carboplatin therapies in dogs with e.g. osteosarcomas at the time of diagnosis or during treatment (Hahn et al., 1996).

At the genomic level the canine *HMGB1* gene exon/intron structure is similar to the human ortholog consisting of five exons and four introns. While the homologies of the exons, lying between 89.4% and 98.7%, are extremely high the homologies of the amplified introns 2 – 4 vary between 20.3% – 59.0%. Approaches to amplify the canine intron 1 resulted in unspecific PCR products which were probably due to the putative existence of various CpG islands found in human intron 1 (Borrmann et al., 2001).

HMGB1 can also be secreted by certain cells, e.g. macrophages (Wang et al. 1999) and as an extracellular protein HMGB1 is a ligand for the receptor for advanced glycation end products (RAGE) (Hori et al., 1995; Parkkinen and Rauvala, 1991; Parkkinen et al., 1993) thus activating key cell signaling pathways, such as p38<sup>MAPK</sup>, JNK, and p42/p44<sup>MAPK</sup>. It plays an important role in inflammation and tumour metastasis regulating growth, invasiveness and motility of tumour cells (Taguchi et al., 2000; Liotta and Clair 2000). Taking into account the previously described importance of the HMGB1-RAGE complex the characterisation of both canine genes *HMGB1* and *RAGE* could serve as basis for studies in dogs aimed at the blocking of the complex leading to a “signal-transduction therapy”.

In human follicular adenomas of the thyroid, structural aberrations of chromosomal band 19q13.4 characterise a large cytogenetic subgroup. Recently, the KRAB zinc finger gene *ZNF331* which is located closely to the breakpoint has been identified as a candidate gene for tumorigenesis of thyroid adenomas (Rippe et al., 1999; Meiboom et al., 2003). As HSA 19q is reported to show homologies to CFA1 (Yang et al., 1999, 2000), which is often affected by aberrations in canine neoplasias (Reimann et al., 1998, 1999), the characterisation of the canine *ZNF331* could reveal if the gene maps to a region frequently affected by chromosomal rearrangements.

The human *ZNF331* (GenBank acc. no. NM018555) is composed of eight exons encoding a KRAB zinc finger protein with only one KRAB-A box. The composed

canine *ZNF331* cDNA (GenBank acc. no. AY375188) consists of 2148 bp showing homologies of 85.3% in the ORF, 39.8% in the 5' UTR, and 35.6% 3' UTR, respective to the human molecule. The deduced canine *ZNF331* protein is a 490 aa protein with a 42 aa KRAB-A box, an 88 aa spacer region, and a 357 aa zinc finger region. The human counterpart is a 463 aa protein with a 42 aa KRAB-A box, an 87 aa spacer region and a 331 aa zinc finger region (Rippe et al., 1999; Wu et al., 2001). On amino acid sequence level the canine *ZNF331* revealed a 92.9% homology for the KRAB-A boxes, 96% for the zinc finger domains, and 59.6% in the spacer region. In the canine *ZNF331* cDNA a 3 bp insertion is found at bp 582-584 leading to an additional arginine in the spacer amino acid sequence. The canine zinc finger domain is extended by 26 amino acids compared to its human counterpart. Recently, seven new human splicing variants were characterised and Northern Blot experiments detected transcripts varying from 2.1 kb to 6.2 kb (Meiboom et al., 2003). Up to date, no screenings for additional canine splicing variants were performed probably due to the fact that thyroid cancer is not very frequent in dogs representing 1.2% - 4.0% of all cancers seen in dogs. On the other hand, 65% - 80% of the canine thyroid neoplasias show malignant behaviour (Withrow and MacEwen, 2001) so the identification of the involved genes could reveal molecular mechanisms inducing malignant behaviour of canine neoplasias.

The known localisation of the human *ZNF331* and the chromosomal assignment of the canine *ZNF331* to CFA 1q33 showed that the genes of both species map to regions affected by chromosomal rearrangements.

The identification of a new candidate gene spanning the chromosomal breakpoint of HSA 2p21 in human thyroid adenomas was done by Rippe et al. (2003). This gene called *THADA* consists of 38 exons coding for a 1663 aa protein. Two fusion products were detected in adenoma cell lines showing fused ectopic sequences downstream of *THADA*'s exon 28. As the breakpoint is the second most frequent structural chromosomal rearrangement seen in benign adenomas of the thyroid (Belge et al., 1998; Bol et al., 1999) the identification of the *THADA* gene opens new perspectives in human thyroid cancer research. Up to date no reports are published finding *THADA* fusions in naturally occurring malignancies. Due to the fact that canine benign thyroid neoplasias are rare, a screening of the malignant canine thyroid cancers for canine *THADA* fusion transcripts could be of value to reveal if these transcripts take part in the process of transformation from adenomas to

carcinomas. A basic characterisation of the canine *THADA* gene would be precondition for such approaches.

Breast cancer in dogs and humans is one of the most frequent malignancies seen in female individuals. In both species the neoplasias are clearly age and hormone dependent (Nolte and Nolte, 2001; Withrow and MacEwen, 2001). One of the genes which is reported to be over-expressed, partially due to gene amplification, in various human cancers including breast cancer is the *Cyclin D1* (*CCND1*) gene, also known as *PRAD1* or *BCL-1*. Due to the similarities of canine and human breast cancer the characterisation of the canine *CCND1* gene could be of great value for new therapeutic approaches.

The cloning of the canine *CCND1* gene allowed the composition of a 1246 bp cDNA contig (GenBank acc. no. AY620434), showing 90.4% sequence identity in the canine ORF, 68.5% in the 3' UTR, and in the 74.7% 5' UTR, respectively, compared to the human counterpart (GenBank acc. no. NM\_053056). The identities of the canine ORF to the ORFs of mouse, rat and chicken (GenBank acc. nos. NM\_007631, NM\_171992, NM\_205381) are 82.4%, 81.3% and 73.8%, respectively. Similar to the human, mouse, and rat orthologue (GenBank acc. nos. NP\_444284, NP\_031657, NP\_741989) the deduced canine protein comprises 295 aa with 93.3%, 89.9% and 88.9% homology to the other species, respectively. The chicken *CCND1* protein (GenBank acc. no. NP\_990712) shows 292 aa with an identity of 86.8% to its canine counterpart. The screened BAC clone containing the canine *CCND1* gene and its mapping to CFA 17 (Meyer et al., 2004a) could be of great value for future studies aiming at the detection of *CCND1* gene amplifications in canine neoplasias.

The described identities of the characterised canine genes to their human counterparts revealed clearly that the sequence conservation is much higher in the protein coding sequences (85% – 95%) than in the UTRs (35% - 95%). These results are in accordance with the results presented by Murua Escobar et al. (2002) for 10.000 canine EST clones showing a much higher conservation of the protein coding sequences. The partially characterized *LHCGR* gene (Exon 11) by Santos et al. (2004) showed the same high identity. The canine exon 11 fragment shared 89%, 89%, 92% and 88% identity to the human, bovine, porcine and murine *LHCGR* sequences respectively.

In this thesis, 13 canine fibrosarcomas, 2 feline fibrosarcomas and 11 canine melanomas were screened for point mutations, particularly within the mutational hot-spot codons of the *KRAS2* and *NRAS* genes, to analyse if these changes could be detected in these naturally occurring tumours.

Point mutations affecting genes of the *ras* family are assumed to be among the most important alterations in human tumourigenesis (Hahn et al., 1994; Arber et al., 1999). All *ras* genes code for 21 kDa proteins which show on one side GTP-GDP-binding activity and on the other side GTPase activity. These proteins play an important role as signal transmitters which are activated by the binding of growth factors initiating cell division by changing the inactive GDP-binding form to an active conformation. This process is reversed by the GTPase activity. Mutations in *ras* genes are assumed to remove the time limit of the cell stimulating signals which results in uncontrolled cell division by affecting the autotermination process (Sigal et al., 1988, 1988a; Singer, 1992; Park, 1995; Watzinger et al., 1998). In malignancies, most amino acid exchanges in *ras* genes are caused by alterations of the so-called hot-spot codons 12, 13, and 61 in exons 1 and 2, respectively, leading to constitutively active *ras* proteins that bring about constant signal transduction, facilitating uncontrolled cell division.

Different studies show that 30% of human lung tumours, 30% of liver tumours, 40% of myelodysplastic syndrome, 30% of acute myelogenous leukaemia, 13% of brain tumours, 53% of follicular and 60% of undifferentiated papillary thyroid tumours, 90% of human pancreatic tumours, and 50% of tumours of the gastrointestinal tract show specific hot spot point mutations in genes of the *ras* gene family (Bos et al., 1989; Knap and Walters, 1997; Tang et al., 2002; Spandidos et al., 2002).

Four of our analysed twentyfour canine and two feline melanoma and fibrosarcoma samples showed nucleotide exchanges in the screened *KRAS2* and *NRAS* exons but none of the found exchanges affected the *ras* hot-spot codons 12, 13, and 61. In canine melanomas so far almost no hotspot *NRAS* mutations were described with one exception: Mayr et al. (2003b) found 2 of 16 melanomas to be affected by mutations in codon 61. In addition Mayr et al. (2003a) showed that *NRAS* mutations were found in canine lymphomas. In contrast to the low mutation frequency seen in dogs, melanoma *NRAS* hot-spot mutation screening in humans detected mutations in up to 33% (Demunter et al., 2001, 2001a; Silvertesson, 2002; Ortonne et al., 2002).

*KRAS2* screening for hot-spot point mutations in dogs has been described in different types of lung tumours, pancreatic cancer and breast cancer like e.g. non small lung cell cancer (Kraegel et al., 1992; Castangnaro, 1995; Tierney et al., 1996; Griffey et al., 1998; Watzinger et al., 2001; Mayr et al., 2003) showing that the canine gene is also affected by the typical *ras* mutations observed in humans but in much lower ratio. Just one report of a *ras* mutation screening including three canine fibrosarcoma samples has been published (Watzinger et al., 2001) and also studies about *KRAS2* mutations in canine melanomas are completely missing.

Our data and the few published data (Kraegel et al., 1992; Castangnaro, 1995; Tierney et al., 1996; Griffey et al., 1998; Watzinger et al., 2001; Mayr et al., 2003, 2003a, 2003b; Murua Escobar et al., in press) strongly suggest that *KRAS2* and *NRAS* mutations at the hotspot loci are very rare and do not play a major role in the pathogenesis of the investigated spontaneously occurring canine melanomas and fibrosarcomas. In contrast to this, *KRAS2* mutations in humans have been described in up to 90% of human pancreatic cancers (Almoguera et al., 1988; Smit et al., 1988; Motojima et al., 1993; Kubrusly et al., 2002; Tada et al., 2002; Yoshizawa et al., 2002), in 17% - 43% of lung tumours (Capon et al., 1983; Ahrendt et al., 2001; Keohavong et al., 2001; Kovalchuk et al., 2001; Liu et al., 2002), in up to 25% of melanomas (Wagner et al., 1995), and in tumours of the gastrointestinal tract as well as in tumours of the skin (Almoguera et al., 1988; Shukal et al., 1989; Belly et al., 2001).

*Ras* genes show high sequence similarity among different mammalian species such as human, cat, dog, cattle, and rodents, with most nucleotide differences representing synonymous changes not affecting the amino acid sequence (Watzinger et al., 1998). We could detect the described point mutations between the canine and feline analysed samples. This detection served also as internal control for specificity of the used DNA amplification method.

The total number of physically mapped canine genes by FISH is up to date (2004) rather small, with fewer than 80 mapped genes (Switonski et al., 2004). Following this, the ten screened and mapped genes in this thesis represent a useful contribution to the canine gene localisation studies. In contrast to localised BAC clones containing known characterised genes the total number of randomly picked localized BAC clones by FISH to canine chromosomes is much higher. In September

2004, Breen et al. mapped 804 BACs which were characterized by BAC end sequencing with an average of 700bp (Breen et al., 2004). The combination of this mapping data combined with the existing RH mapping data and future BAC characterisations could be of great value for future studies of the canine genome.

Finally, for molecular studies in all species a critical point is the quality of the provided tissues and the method of tissue fixation. Commonly used formalin-fixed, paraffin embedded (FFPE) tissue material for pathologic and molecular pathologic applications is often not suited for downstream applications, such as high molecular DNA and RNA isolation. The HOPE-(Hepes-glutamic acid buffer mediated Organic solvent Protection Effect) technique comprises a new protection-solution allowing pathologic routine investigations, i.e., tissue fixation, subsequent paraffin-embedding, and sectioning (Olert et al., 2001) Part of the work of this thesis was participation in investigations to evaluate if RNA from HOPE-fixed tissue samples is suitable for Northern Blot and microarray analyses. The results obtained clearly showed that RNA from HOPE-fixed tissues is suitable for Northern Blot and microarray analyses (Goldmann et al., 2004) offering a useful alternative to the established standard fixation methods.

Summarising, the characterisation of the canine genome and especially of the cancer and disease related genes will be of great value for the development of new therapeutic approaches for both species dogs and humans. The similarities found in the candidate genes and proteins additional to the described similarities of the biological behaviour seen in different cancers and diseases of both species offer the opportunity of establishing a new model system with various advantages compared to other model organisms e.g. in view of analysing disease related molecular mechanisms, drug development and evaluation, and transferability of the therapeutic approaches from the model system to humans.

## 5 Summary

The canine genome offers a wide field for genetic studies on various areas like e.g. phenotypic diversity, heredity and diseases including cancer. The diversity of the different canine breeds in terms of phenotype and behaviour is unique within mammals. Currently, the “Canine Genetic Disease Information System” contains the clinical, pathological and genetic features of more than 370 genetic disorders. This constitutes the largest set of naturally occurring genetic disorders in any non-human species. More than 50% of the recognised canine genetic diseases resemble the specific human genetic diseases and more than 46% of the identified genetic disorders occur predominantly in one or a few breeds.

In terms of cancer the dog shows additional advantages. The cancers seen in pet dogs are spontaneously developing as opposed to cancers of laboratory rodents. This suggests that the mechanisms of development in canine tumours could be comparable to humans. It is generally believed that dogs develop cancer twice as frequently as humans, and that the presentation, histology and biology of several canine cancers is similar to humans. Most canine cancers progress more rapidly than their human counterparts permitting a better monitoring of the tumour state. The canine cancers are more akin to human cancers than rodent tumours in terms of patient size and cell kinetics allowing better comparison of medical examinations like e.g. ultrasonography. Additionally, dogs show similar characteristics of physiology and metabolism for most organ systems and drugs, which allows better comparability of modalities e.g. surgery, radiation and chemotherapy.

However, precondition for using the dog as model system for human diseases including cancer is the knowledge of the canine genes involved. In this thesis, the canine *HMGA1*, *HMGB1*, *ZNF331*, and *CCND1* genes were characterised on various levels including their cDNA structure, partial genomic DNA structure, and their expression patterns. Their corresponding proteins were predicted by *in silico* analyses and gene identity analyses were carried out comparing these characterised genes with their corresponding counterparts of various other species. Additionally, the gene loci of these genes and of six other genes were defined by FISH analyses.

Further on, to analyse if known molecular mechanisms involved in human cancers are existent in dogs as well, thirteen canine fibrosarcomas, two feline fibrosarcomas, and eleven canine melanomas were screened for hot-spot mutations of the *NRAS*

and *KRAS2* genes. No such mutations could be detected, suggesting that these mutations do not play a major role in the analysed neoplasias.

Additionally, in a study involving the human *ZNF331* gene new splicing variants could be detected and it was participated in a study identifying and characterising a new candidate gene (*THADA*) for human thyroid neoplasias. Finally, the evaluation of the new fixative HOPE for molecular methods was carried out.

## 6 References

Abe N, Watanabe T, Masaki T, Mori T, Sugiyama M, Uchimura H, Fujioka Y, Chiappetta G, Fusco A. and Atomi Y. (2000): Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. *Cancer Res* 60: 3117-22.

Abe N, Watanabe T, Sugiyama M, Uchimura H, Chiappetta G, Fusco A and Atomi, Y. (1999): Determination of high mobility group I(Y) expression level in colorectal neoplasias: a potential diagnostic marker. *Cancer Res* 59: 1169-74.

Ahrendt SA, Decker P, Alawi E, Zhu Y, Sanchez-Cespedes M, Yang SC, Haasler GB, Kajdacsy-Balla A; Demeure MJ, Sidransky D. (2001): Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. *Cancer* 92: 1525-30

Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. (1988): Most human carcinomas of the exocrine pancreas contain mutant *c-K-ras* genes. *Cell* 53: 549-554

Arber N. (1999): Janus faces of ras: anti or pro-apoptotic? *Apoptosis*: 4(5):3838.

Bandiera A, Bonifacio D, Manfioletti G, Mantovani F, Rustighi A, Zanconati F, Fusco A, Di Bonito L and Giancotti V. (1998): Expression of HMGI(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. *Cancer Res* 58: 426-31.

Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, Bullerdiek J. (2003): The canine HMGA1 gene maps to CFA 23. *Anim Genet.* 34(1): 68-9

Belge G, Roque L, Soares J, Bruckmann S, Thode B, Fonseca E, Clode A, Bartnitzke S, Castedo S and Bullerdiek J. (1998): Cytogenetic investigation of 340 thyroid hyperplasias and adenomas revealing correlations between cytogenetic findings and histology. *Cancer Genet. Cytogenet.*, 101: 42–8.

Belly RT, Rosenblatt JD, Steinmann M, Toner J, Sun J, Shehadi J, Peacock JL, Raubertas RF, Jani N, Ryan CK. (2001): Detection of mutated K12-ras in histologically negative lymph nodes as an indicator of poor prognosis in stage II colorectal cancer. *Clin. Colorectal Cancer* 1: 110-6

Bol S, Belge G, Rippe V, Bullerdiek J. (2001): Molecular cytogenetic investigation define a subgroup of thyroid adenomas with 2p21 breakpoints clustered to a region of less than 450 kb. *Cytogenet. Cell Genet.* 95, 189–91

von Bomhard, D. (2001): Epidemiologie. In: Nolte, I, Nolte, M (eds), - Praxis der Onkologie bei Hund und Katze. Stuttgart, Enke Verlag.

Boonyaratanakornkit V, Melvin V, Prendergast P, Altmann M, Ronfani L, Bianchi ME, Taraseviciene L, Nordeen SK, Allegretto EA, Edwards DP. (1998): High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. *Mol Cell Biol* 18: 4471-87

Borrmann L, Schwanbeck R, Heyduk T, Seebeck B, Rogalla P, Bullerdiek J, Wisniewski JR. (2003): High mobility group A2 protein and its derivatives bind a specific region of the promoter of DNA repair gene ERCC1 and modulate its activity. *Nucleic Acids Res.* 31:6841-51.

Bos JL. (1989): Ras oncogenes in human cancer: a review. *Cancer Res.* **50**: 1352

Breen M, Thomas R, Binns MM, Carter NP, Langford CF. (1999a): Reciprocal Chromosome Painting Reveals Detailed Regions of Conserved Synteny between the Karyotypes of the Domestic Dog (*Canis familiaris*) and Human. *Genomics* 61: 145-55

Breen M, Bullerdiek J, Langford CF. (1999b): The DAPI banded karyotype of the domestic dog (*Canis familiaris*) generated using chromosome-specific paint probes *Chromosome Res.* 7(5): 401-6.

Breen M, Langford CF, Carter NP, Holmes NG, Dickens HF, Thomas R, Suter N, Ryder EJ, Pope M, Binns MM. (1999c): FISH mapping and identification of canine chromosomes. *J Hered.* 90(1): 27-30.

Breen M, Jouquand S, Renier C, Mellersh CS, Hitte C, Holmes NG, Cheron A, Suter N, Vignaux F, Bristow AE, Priat C, McCann E, Andre C, Boundy S, Gitsham P, Thomas R, Bridge WL, Spriggs HF, Ryder EJ, Curson A, Sampson J, Ostrander EA, Binns MM, Galibert F. (2001): Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid/linkage map of the domestic dog genome to all chromosomes. *Genome Res.* 11(10): 1784-95.

Breen M, Hitte C, Lorentzen TD, Thomas R, Cadieu E, Sabacan L, Scott A, Evanno G, Parker HG, Kirkness E, Hudson R, Guyon R, Mahairas GG, Gelfenbeyn B, Fraser CM, Andre C, Galibert F, Ostrander EA. (2004): An integrated 4249 marker FISH/RH map of the canine genome. *BMC Genomics.* 5(1):65.

Bustin M, Reeves R. (1996): High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. *Prog Nucleic Acid Res Mol Biol.* 54: 35-100.

Bustin M. (2001): Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends Biochem Sci* 26(3): 152-3.

Capon DJ, Seeburg PH, McGrath JP, Hayflick JS, Edman U, Levinson AD, Goeddel DV. (1983): Activation of Ki-ras2 gene human colon and lung carcinomas by two different point mutations. *Nature* 304: 507-513

Castagnaro M. (1995): Ras gene analysis in mammary tumors of dogs by means of PCR-SSCP and direct genomic analysis. *Ann Ist Super Sanita*, 31(3):337-41.

Chau KY, Lam HY, Lee KL.(1998): Estrogen treatment induces elevated expression of HMG1 in MCF-7 cells. *Exp Cell Res* 241: 269-272

Chuvpilo S, Schomberg C, Gerwig R, Heinfling A, Reeves R, Grummt F, Serfling E. (1993): Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter. *Nucleic Acids Res.* 21(24): 5694-704.

Chiappetta G, Bandiera A, Berlingieri MT, Visconti R, Manfioletti G, Battista S, Martinez-Tello FJ, Santoro M, Giancotti V and Fusco A. (1995): The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasias. *Oncogene* 10: 1307-14.

Chiappetta, G., Avantaggiato, V., Visconti, R., Fedele, M., Battista, S., Trapasso, F., Merciai, B.M., Fidanza, V., Giancotti, V., Santoro, M., Simeone, A. and Fusco, A., (1996): High level expression of the HMGI (Y) gene during embryonic development. *Oncogene* 13: 2439-46.

Chiappetta, G., Tallini, G., De Biasio, M.C., Manfioletti, G., Martinez-Tello, F.J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giancotti, V. and Fusco, A., (1998): Detection of high mobility group I HMGI(Y) protein in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma. *Cancer Res* 58: 4193-8.

Czyz W, Balcerczak E, Jakubiak M, Pasięka Z, Kuzdak K, Mirowski M. (2004): HMGI(Y) gene expression as a potential marker of thyroid follicular carcinoma. *Langenbecks Arch Surg.* 389(3):193-7

Das D, Scovell WM. (2001): The binding interaction of HMG-1 with the TATA-binding protein/TATA complex. *J Biol Chem* 276: 32597-605

Demunter A, Ahmadian MR, Libbrecht L, Stas M, Baens M, Scheffzek K, Degreeef H, De Wolf-Peeters C, van den Oord JJ. (2001): A novel N-ras mutation in malignant melanoma is associated with excellent prognosis. *Cancer Res.* 61: 4916-22

Demunter A, Stas M, Degreeef H, de Wolf-Peeters C, van den Oord JJ. (2001a): Analysis of N- and K-ras mutations in the distinctive tumor progression phases of melanoma. *Invest. Dermatol.* 117: 1483-89

Du W, Thanos D, Maniatis T. (1993): Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. *Cell* 74: 887-98

Du W, Maniatis, T. (1994): The high mobility group protein HMG I(Y) can stimulate or inhibit DNA binding of distinct transcription factor ATF-2 isoforms. *Proc.Natl.Acad.Sci.U.S.A* 91: 11318-22.

Fedele M, Bandiera A, Chiappetta G, Battista S, Viglietto G, Manfioletti G, Casamassimi A, Santoro M, Giancotti V and Fusco A. (1996): Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. *Cancer Res* 56: 1896-901.

Ferrari S, Ronfani L, Calogero S, Bianchi ME. (1994): The mouse gene coding for high mobility group 1 protein (HMG1). *J Biol Chem* 269:28803-08

Ge H, Roeder RG: The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein. (1994): *J Biol Chem* 269: 17136-140

Goldmann T, Flohr AM, Murua Escobar H, Gerstmayer B, Janssen U, Bosio A, Loeschke S, Vollmer E, Bullerdiek J. (2004): The HOPE-technique permits Northern blot and microarray analyses in paraffin-embedded tissues. *Pathol. Res. Prac.* 200: 511-5 .

Griffey SM, Kraegel SA, Madewell BR (1998): Rapid detection of K-ras gene mutations in canine lung cancer using single-strand conformational polymorphism analysis. *Carcinogenesis* 19: 959-63

Guerrero S, Figueras A, Casanova I, Farre L, Lloveras B, Capella G, Trias M, Mangués R. (2002): Codon 12 and codon 13 mutations at the K-ras gene induce different soft tissue sarcoma types in nude mice. *Faseb J*, 16(12):1642-4.

Guyon R, Lorentzen TD, Hitte C, Kim L, Cadieu E, Parker HG, Quignon P, Lowe JK, Renier C, Gelfenbeyn B, Vignaux F, DeFrance HB, Gloux S, Mahairas GG, Andre C, Galibert F, Ostrander EA. (2003): A 1-Mb resolution radiation hybrid map of the canine genome. *Proc Natl Acad Sci U S A*. 100(9): 5296-301.

Hahn KA, Bravo L, Adams WH, Frazier DL. (1994): Naturally occurring tumors in dogs as comparative models for cancer therapy research. *In Vivo*. 8(1): 133-43.

Hahn KA, Legendre AM and Talbott JR. (1996): The frequency of micronuclei in lymphocytes of dogs with osteosarcomas: a predictive variable for tumor response during cisplatin chemotherapy. *Cancer Epidemiol Biomarkers Prev* 5: 653-6.

He Q, Liang CH, Lippard SJ. (2000): Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. *Proc Natl Acad Sci U S A* 97:5768-72

Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, Morser J, Stern D, Schmidt AM. (1995): The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphotericin. Mediation of neurite outgrowth and co-expression of rage and amphotericin in the developing nervous system. *J Biol Chem* 270: 25752-61

John S, Reeves RB, Lin JX, Child R, Leiden JM, Thompson CB, Leonard WJ. (1995): Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between E1f-1, HMG-I(Y), and NF-kappa B family proteins. *Mol. Cell Biol*. 15: 1786-96.

John S, Robbins CM, Leonard WJ. (1996). An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, E1f-1, HMG-I(Y) and a GATA family protein. *EMBO J*. 15: 5627-35

Kazmierczak B, Dal Cin P, Wanschura S, Borrmann L, Fusco A, Van den Berghe H and Bullerdiek J. (1998): HMG1Y is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. *Genes Chromosomes Cancer* 23: 279-85.

Keohavong P, Mady HH, Gao WM, Siegfried JM, Luketich JD, Melhem MF. (2001): Topographic analysis of K-ras mutations in histologically normal lung tissues and tumors of lung cancer patients. *Br. J. Cancer* 20: 235-41

Klein-Hessling S, Schneider G, Heinfling A, Chuvpilo S, Serfling E. (1996): HMG I(Y) interferes with the DNA binding of NF-AT factors and the induction of the interleukin 4 promoter in T cells. *Proc.Natl.Acad.Sci.U.S.A* 93: 15311-6.

Knapp DW and Waters DJ, (1997): Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. *Mol Med Today*. 1997 3(1): 8-11.

Kovalchuk O, Chyczewska E, Niklinska W, Niklinski J, Naumnik W, Chyczewski L. (2001): K-ras codon 12 mutations detected with enriched PCR method in operable non-small cell lung cancer. *Folia. Histochem. Cytobiol.* 39: 68-9

Kraegel SA, Gumerlock PH, Dungworth DL, Oreffo VIC., Madewell BR. (1992): K-ras activation in non-small cell lung cancer in the dog. *Cancer Res.* 52: 4724-7

Kubrusly MS, Cunha JE, Bacchella T, Abdo EE, Jukemura J, Penteadó S, Morioka CY, de Souza LJ, Machado MC. (2002): Detection of K-ras mutation at codon 12 in pancreatic diseases: a study in a Brazilian casuistic. *J.O.P.* 3: 144-151

Lewis, H., Kaszubska, W., DeLamarter, J. F., Whelan, J. (1994): Cooperativity between two NF-kappa B complexes, mediated by high-mobility-group protein I(Y), is essential for cytokine-induced expression of the E-selectin promoter. *Mol.Cell Biol.* 14: 5701-9

Li R, Mignot E, Faraco J, Kadotani H, Cantanese J, Zhao B, Lin X, Hinton L, Ostrander EA, Patterson DF, de Jong PJ. (1999): Construction and characterization of an eightfold redundant dog genomic bacterial artificial chromosome library. *Genomics.* 58(1): 9-17.

Lingaas F, Sørensen A, Juneja KR, Johansson S, Fredholm M, Winterø AK, Sampson J, Mellersh C, Curzon A, Holmes NG, Binns MM, Dickens HF, Ryder EJ, Gerlach J, Baumle E, Dolf G. (1997): Towards construction of a canine linkage map: establishment of 16 linkage groups. *Mamm. Genome* 8: 218–21

Liotta LA, Clair T: (2000): Cancer. Checkpoint for invasion. *Nature* 405: 287-8

Liu JB, Xie JP, Zou ZL, Chen LJ, Li LY, Wang SQ. (2002): Detection of P53 and K-ras gene mutations in lung cancer with oligonucleotide chip. *Sheng Wu Gong Xue Bao* 18: 447-51

MacEwen EG, (1990): Spontaneous tumours in dogs and cats: models for the study of cancer biology and treatment. *Cancer Metastasis Rev.* 9: 125-36

Mayr B, Schaffner G, Reifinger M. (2003): K-ras mutations in canine pancreatic cancers. *Vet Rec* 153(3):87-9.

Mayr B, Holzheu M, Schaffner G, Reifinger M. (2003a): N-ras mutation in a canine lymphoma: short communication. *Acta Vet Hung.* 51(1): 91-4.

Mayr B, Schaffner G, Reifinger M, Zwetkoff S, Prodinger B. (2003b): N-ras mutations in canine malignant melanomas. *Vet J.* 165(2):169-71.

Meiboom M, Murua Escobar H, Pentimalli F, Fusco A, Belge G, Bullerdiek J. (2003): A 3.4-kbp transcript of ZNF331 is solely expressed in follicular thyroid adenomas. *Cytogenet Genome Res.* 101(2): 113-7

Meiboom M, Murua Escobar H, Winkler S, Nolte I, Bullerdiek J. (2004): Molecular characterization and mapping of the canine KRAB zinc finger gene ZNF331. *Anim Genet* 35(3): 262-3.

Mellersh CS, Langston AA, Acland GM, Fleming MA, Ray K, Wiegand NA, Francisco LV, Gibbs M, Aguirre GD, Ostrander EA. (1997): A linkage map of the canine genome. *Genomics.* 46(3): 326-36.

Mellersh CS, Hitte C, Richman M, Vignaux F, Priat C, Jouquand S, Werner P, Andre C, DeRose S, Patterson DF, Ostrander EA, Galibert F. (2000): An integrated linkage-radiation hybrid map of the canine genome. *Mamm Genome*. 11(2): 120-30.

Meyer B, Murua Escobar H, Winkler S, Richter A, Schelling C, Dolf G, Nolte I, Bullerdiek J. (2004a): Molecular characterization and mapping of the canine Cyclin D1 (CCND1) gene. *Anim Genet*. 35(5): 413

Meyer B, Murua Escobar H, Hauke S, Richter A, Winkler S, Rogalla P, Flohr AM, Bullerdiek J, Nolte I. (2004): Expression pattern of the HMGB1 gene in sarcomas of the dog. *Anticancer Res* 24(2B): 707-10

Meyer J, Murua Escobar H, Winkler S, Schelling C, Dolf G, Nolte I, Bullerdiek J. (in press): The FAS-activated serine/threonine kinase (FASTK) gene maps to canine chromosome 16. *Anim Genet*.

Motojima K, Urano T, Nagata Y, Shiku H, Tsurifune T, Kanematsu T. (1993): Detection of point mutations in the Kirsten-ras oncogene provides evidence for the multicentricity. *Ann. Surg.* 217: 138-143

Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME. (2001): New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J.*, 15; 20(16): 4337-40

Murua Escobar H, Becker K, Bullerdiek J, Nolte I. (2001): The canine ERBB2 gene maps to a chromosome region frequently affected by aberrations in tumors of the dog (*Canis familiaris*). *Cytogenet Cell Genet*. 94(3-4): 194-5

Murua Escobar H, Borrmann L, Nimzyk R, Bullerdiek J, Nolte I. (2002): Characterization of 10,000 canine ESTs. Proceedings of the 28th International Conference on Animal Genetics, Goettingen 11-15 August 2002, abstract A029, ISBN 3-00-010597-2, <http://www.isag.org.uk/conference2000.htm>

Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM, Bullerdiek J, Nolte I. (2003): Molecular characterization of the canine HMGB1. *Cytogenet Genome Res.* 101(1): 33-8

Murua Escobar H, Meyer J, Winkler S, Schelling C, Dolf G, Nolte I Bullerdiek J. (2004): The protein kinase B, gamma (AKT3) gene maps to canine chromosome 7 *Anim Genet.* 35(4): 354-5

Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, Bullerdiek J. (2004a) The canine HMGA1. *Gene* 14(330): 93-9.

Murua Escobar H, Günther K, Richter A, Soller JT, Winkler S, Nolte I, Bullerdiek J. (in press): Absence of ras-gene hotspot mutations in canine fibrosarcomas and melanomas. *Anticancer Res.*

Neff MW, Broman KW, Mellersh CS, Ray K, Acland GM, Aguirre GD, Ziegler JS, Ostrander EA, Rine J. (1999): A second-generation genetic linkage map of the domestic dog, *Canis familiaris*. *Genetics.* 151(2): 803-20.

Nolte, I, Nolte, M. (2000): *Praxis der Onkologie bei Hund und Katze.* Stuttgart, Enke Verlag.

Olert J, Wiedorn KH, Goldmann T, Kühl H, Mehraein Y, Scherthan H, Niketeghad F, Vollmer E, Müller AM, Müller-Navia J. (2001): HOPE-fixation: A novel fixing method and paraffin embedding technique for human soft tissues. *Pathol Res Pract* 197:823-6

Ortonne JP. (2002): Photobiology and genetics of malignant melanoma. *Br. J. Dermatol.* 146: 11-16

Ostrander EA, Galibert F, Patterson DF. (2000): Canine genetics comes of age. *Trends Genet.* 16: 117-24.

Paonessa G, Frank R, Cortese R. (1987): Nucleotide sequence of rat liver HMG1 cDNA. *Nucleic Acids Res* 15: 9077

Park M. (1995): Genetic Abnormalities of Cell Growth, in Scriver et al.: The Metabolic and Molecular Basis of Inherited Disease, 7 edn. New York: McGraw-Hill.

Parkkinen J, Rauvala H. (1991): Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin: Enhancement of t-PA-catalyzed plasminogen activation by amphoterin. *J Biol Chem* 266: 16730-5

Parkkinen J, Raulo E, Merenmies J, Nolo R, Kajander EO, Baumann M, Rauvala H. (1993): Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. *J Biol Chem* 268: 19726-38

Patterson DF, Haskins ME, Jezyk PF. (1982): Models of human genetic disease in domestic animals. *Adv Hum Genet.* 12: 263-339

Patterson DF, Haskins ME, Jezyk PF, Giger U, Meyers-Wallen VN, Aguirre G, Fyfe JC, Wolfe JH. (1988): Research on genetic diseases: reciprocal benefits to animals and man. *J Am Vet Med Assoc.* 193(9): 1131-44

Patterson DF. (2000a): Canine Genetic Information System: A Computerized Knowledgebase of Genetic Diseases in Dogs

Patterson DF. (2000b): Companion animal medicine in the age of medical genetics. *J. Vet. Internal Med.* 14: 1-9

Pil PM, Lippard SJ (1992): Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science* 256: 234-7

Priat C, Hitte C, Vignaux F, Renier C, Jiang Z, Jouquand S, Cheron A, Andre C, Galibert F. (1998): A whole-genome radiation hybrid map of the dog genome. *Genomics.* 54(3): 361-78.

Reeves R and Beckerbauer L. (2001): HMGI/Y proteins: flexible regulators of transcription and chromatin structure. *Biochim Biophys Acta* 1519: 13-29.

Reeves R, Edberg DD and Li Y. (2001): Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. *Mol Cell Biol* 21: 575-94.

Reimann, N, Bartnitzke, S, Bullerdiek, J, Schmitz, U, Rogalla, P, Nolte, I, Ronne, M, (1996): An extended nomenclature of the canine. *Cytogenet Cell Genet* 73: 140-4.

Reimann N, Bartnitzke S, Bullerdiek J, Mischke R, Nolte I. (1998): Trisomy 1 in a canine acute leukemia indicating the pathogenetic importance of polysomy 1 in leukemias of the dog. *Cancer Genet Cytogenet* 101: 49–52.

Reimann N, Nolte I, Bullerdiek J. (1999): Cancer genetics going to the dogs: The future has begun. *J natl Cancer Inst* 91:1688–9.

Richter A, Murua Escobar H, Günther K, Meyer B, Winkler S, Dolf G, Schelling C, Nolte I Bullerdiek J. (2004): The canine NRAS gene maps to CFA 17. *Anim Genet.* 35(4): 355-6

Rippe V, Drieschner N, Meiboom M, Murua Escobar H, Bonk U, Belge G, Bullerdiek J. (2003): Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas. *Oncogene.* 22(38): 6111-4.

Rogalla P, Drechsler K, Frey G, Hennig Y, Helmke B, Bonk U, Bullerdiek J. (1996): HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors. *Am J Pathol.* 149(3): 775-9.

Romine LE, Wood JR, Lamia LA, Prendergast P, Edwards DP, Nardulli AM: The high mobility group protein 1 enhances binding of the estrogen receptor DNA binding domain to the estrogen response element. (1998): *Mol Endocrinol* 12: 664-74

Santos SE, Murua Escobar H, Sider LH, Winkler S, Aoki SM, Milazzotto MP, Campagnari F, Vannucchi CI, Bullerdiek J, Nolte I, Garcia JF. (2004): DNA sequence, polymorphism, and mapping of luteinizing hormone receptor fragment (LHCGR) gene in Great Dane dogs. *Anim Genet.* 35(1): 74-75

Scala S, Portella G, Fedele M, Chiappetta G and Fusco A. (2000): Adenovirus-mediated suppression of HMGI(Y) protein synthesis as potential therapy of human malignant neoplasias. *Proc Natl Acad Sci U S A* 97: 4256-61.

Scott J. and Fuller J. (1965): *Genetics and the Social Behaviour of the Dog*, University of Chicago Press

Selden JR, Moorhead PS, Oehlert ML, Patterson DF. (1975): The Giemsa banding pattern of the canine karyotype. *Cytogenet Cell Genet.* 15(6): 380-7.

Shukla SK, Hughes DC, Hughes LE, McCormick F, Padua RA. (1989): Ras mutations in human melanotic lesions: K-ras activation is a frequent and early event in melanoma development. *Oncogene Res.* 5: 121-127

Sigal IS, D'Alonzo JS, Ahern JD, Marshall MS, Smith GM, Scolnick EM, Gibbs JB: (1988): The ras oncogene protein as a G-protein. *Adv. Second Messenger Phosphoprotein Res.* 21: 193-200

Sigal IS, Marshall MS, Schaber MD, Vogel US, Scolnick EM, Gibbs JB. (1988a): Structure/ function studies of the ras protein. *Cold Spring Harb. Symp. Quant. Biol.* 53: 863-9

Singer M.(1992): The ras gene and cancer. Symposium transcript from the University of California, San Francisco: [www.accessexcellence.org](http://www.accessexcellence.org)

Sivertsson A, Platz A, Hansson J, Lundeberg J. (2002): Pyrosequencing as an alternative to single-strand conformation polymorphism analysis for detection of N-ras mutations in human melanoma metastases. *Clin. Chem.* 48: 2164-70

Smit VT, Boot AJ, Smits AM, Fleuren GJ, Cornelisse CJ, Bos JL. (1988): KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nucleic Acids Res.* 16: 7773-7782

Spandidos DA, Sourvinos G, Tsatsanis C, Zafiropoulos A.. (2002): Normal ras genes: their onco-suppressor and pro-apoptotic functions (review). *Int J Oncol* 21(2): 237-41

Switonski M, Reimann N, Bosma AA, Long S, Bartnitzke S, Pienkowska A, Moreno-Milan MM, Fischer P. (1996): Report on the progress of standardization of the G-banded canine (*Canis familiaris*) karyotype. Committee for the Standardized Karyotype of the Dog (*Canis familiaris*). *Chromosome Res* 4: 306-9.

Switonski M, Szczerbal I, Nowacka J. (2004): The dog genome map and its use in mammalian comparative genomics. *J. Appl. Genet.* 45(2): 195-214

Tada M, Tateishi K, Kawabe T, Sasahira N, Isayama H, Komatsu Y, Shiratori Y, Omata M. (2002): Quantity of mutant K-ras gene in pancreatic secretions for diagnosis of pancreatic carcinoma with different assays: analysis of 100 patients. *Clin. Chim. Acta.* 324: 105-11

Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM. (2000): Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 405: 354-60

Takaha N, Resar LM, Vindivich D, Coffey DS. (2004): High mobility group protein HMGI(Y) enhances tumor cell growth, invasion, and matrix metalloproteinase-2 expression in prostate cancer cells. *Prostate.* 60(2):160-7.

Tallini G, Vanni R, Manfioletti G, Kazmierczak B, Faa G, Pauwels P, Bullerdiek J, Giancotti V, Van Den Berghe H and Dal Cin, P. (2000): HMGI-C and HMGI(Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroid hamartomas, endometrial polyps, and uterine leiomyomas and is compatible with rearrangement of the HMGI-C and HMGI(Y) genes. *Lab Invest* 80: 359-69.

Tamimi Y, van der Poel HG, Denyn MM, Umbas R, Karthaus HF, Debruyne FM and Schalken JA. (1993): Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization. *Cancer Res* 53: 5512-6.

Tang EM. (2002): Carcinogen in cigarettes causes gene mutation. *J. Natl. Cancer Inst.* 94: 1527-36

Thanos D. und Maniatis T. (1992): The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene. *Cell* 71: 777-89

Thanos D. und Maniatis T. (1995a): NF-kappa B: a lesson in family values. *Cell* 80: 529-32

Tierney LA, Hahn FF, Lechner JF. (1996): p53, erbB-2 and K-ras gene alterations are rare in spontaneous and plutonium-239-induced canine lung neoplasia. *Radiat Res* 145(2):181-7

Verrier CS, Roodi N, Yee CJ, Bailey LR, Jensen RA, Bustin M, Parl FF. (1997): High-mobility group (HMG) protein HMG-1 and TATA-binding protein-associated factor TAF(II)30 affect estrogen receptor-mediated transcriptional activation.: *Mol Endocrinol* 11: 1009-19

Wagner SN, Ockenfels HM, Wagner C, Hofler H, Goos M. (1995): Ras gene mutations: a rare event in nonmetastatic primary malignant melanoma. *Invest. Dermatol.* 104: 868-71

Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. (1999): HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 285: 248-51

Watzinger F., Mayr B, Haring E, Lion T. (1998): High sequence similarity within ras exons 1 and 2 in different mammalian species and phylogenetic divergence of the ras gene family. *Mamm. Genome* 9: 214-9

Watzinger F, Mayr B, Gamerith R, Vetter C, Lion T. (2001): Comparative analysis of ras proto-oncogene mutations in selected mammalian tumors. *Mol. Carcinog.* 30: 190-8

Wayne RK. (1993): Molecular evolution of the dog family. *Trends Genet.*: 218-24

Wayne RK, Ostrander EA. (2004): Out of the dog house: the emergence of the canine genome. *Heredity* 92(4):273-4

Williams AJ, Powell WL., Collins T. and Morton CC. (1997): HMGI(Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm. *Am J Pathol* 150: 911-8.

Winkler S, Murua Escobar H, Günther K, Richter A, Dolf G, Schelling C, Bullerdiek J, Nolte I. (2004): The canine KRAS2 gene maps to CFA 22. *Anim Genet.* 35(4): 350-1.

Withrow SJ and MacEwen EG. (1989): *Clinical Veterinary Oncology*, J.B. Lippincott, Co.

Withrow SJ and MacEwen EG. (2001): *Small Animal Clinical Oncology*, W.B. Saunders Company

Yang F, O'Brien PC, Milne BS, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D & Ferguson-Smith MA. (1999): A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. *Genomics* 62, 189-202.

Yang F, Milne BS, Schelling C, Dolf G, Schlapfer J, Switonski M, Ladon D, Pienkowska A, Bosma AA, Sargan DR, Ferguson-Smith MA. (2000): Chromosome identification and assignment of DNA clones in the dog using a red fox and dog comparative map. *Chromosome Res.* 8(2): 93-100.

## *References*

---

Yie J, Liang S, Merika M, Thanos D. (1997): Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter. *Mol.Cell Biol.* 17: 3649-62.

Yoshizawa K, Nagai H, Sakurai S, Hironaka M, Morinaga S, Saitoh K, Fukayama M. (2002): Clonality and K-ras mutation analyses of epithelia in intraductal papillary mucinous tumor and mucinous cystic tumor of the pancreas. *Virchows Arch.* 441: 437-43

Zappavigna V, Falciola L, Helmer-Citterich M, Mavilio F, Bianchi ME: HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. (1996): *EMBO J* 15: 4981-91

## **7 Publications in Reverse Chronological Order**

### ***I. Murua Escobar H, Günther K, Richter A, Soller JT, Winkler S, Nolte I, Bullerdiek J***

Absence of ras-gene hotspot mutations in canine fibrosarcomas and melanomas  
Anticancer Res (in press)

### ***II. Meyer B, Murua Escobar H, Winkler S, Richter A, Schelling C, Dolf G, Nolte I, Bullerdiek J***

Molecular characterization and mapping of the canine Cyclin D1 (*CCND1*) gene  
Anim Genet. 2004; 35(5):413

### ***III. Meyer J, Murua Escobar H, Winkler S, Schelling C, Dolf G, Nolte I, Bullerdiek J***

The FAS-activated serine/threonine kinase (*FASTK*) gene maps to canine chromosome 16

Anim Genet (submitted)

### ***IV. Murua Escobar H, Meyer J, Winkler S, Schelling C, Dolf G, Nolte I Bullerdiek J***

The protein kinase B, gamma (*AKT3*) gene maps to canine chromosome 7

Anim Genet. 2004; 35(4): 354-5

### ***V. Richter A, Murua Escobar H, Günther K, Meyer B, Winkler S, Dolf G, Schelling C, Nolte I Bullerdiek J***

The canine *NRAS* gene maps to CFA 17

Anim Genet. 2004; 35(4): 355-6

### ***VI. Winkler S, Murua Escobar H, Günther K, Richter A, Dolf G, Schelling C, Bullerdiek J, Nolte I***

The canine *KRAS2* gene maps to CFA 22

Anim Genet. 2004; 35(4): 350-1

**VII. Meiboom M, Murua Escobar H, Winkler S, Nolte I, Bullerdiek J**

Molecular characterization and mapping of the canine KRAB zinc finger gene *ZNF331*

Anim Genet. 2004; 35(3): 262-3

**VIII. Meyer B, Murua Escobar H, Hauke S, Richter A, Winkler S, Rogalla P, Flohr AM, Bullerdiek J, Nolte I**

Expression pattern of the *HMGB1* gene in sarcomas of the dog

Anticancer Res. 2004; 24(2B): 707-10

**IX. Goldmann T, Flohr AM, Murua Escobar H, Gerstmayer B, Janssen U, Bosio A, Loeschke S, Vollmer E, Bullerdiek J**

The HOPE-technique permits Northern blot and microarray analyses in paraffin-embedded tissues

Pathol Res Prac. 2004; 200: 511-5

**X. Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, Bullerdiek J.**

The canine *HMGA1*

Gene. 2004; 14(330): 93-9

**XI. Santos SE, Murua Escobar H, Sider LH, Winkler S, Aoki SM, Milazzotto MP, Campagnari F, Vannucchi CI, Bullerdiek J, Nolte I, Garcia JF.**

DNA sequence, polymorphism, and mapping of luteinizing hormone receptor fragment (*LHCGR*) gene in Great Dane dogs

Anim Genet. 2004; 35(1): 74-5

**XII. Meiboom M, Murua Escobar H, Pentimalli F, Fusco A, Belge G, Bullerdiek J.**

A 3.4-kbp transcript of *ZNF331* is solely expressed in follicular thyroid adenomas

Cytogenet Genome Res. 2003; 101(2): 113-7

**XIII. Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM, Bullerdiek J, Nolte I.**

Molecular characterization of the canine *HMGB1*  
Cytogenet Genome Res. 2003; 101(1): 33-8

**XIV. Rippe V, Drieschner N, Meiboom M, Murua Escobar H, Bonk U, Belge G, Bullerdiek J.**

Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas  
Oncogene. 2003; 22(38): 6111-4

**XV. Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, Bullerdiek J.**

The canine *HMGA1* gene maps to CFA 23.  
Anim Genet. 2003; 34(1): 68-9

**XVI. Murua Escobar H, Becker K, Bullerdiek J, Nolte I**

The canine *ERBB2* gene maps to a chromosome region frequently affected by aberrations in tumors of the dog (*Canis familiaris*)  
Cytogenet Cell Genet. 2001; 94(3-4): 194-5

# I.

## **Absence of *ras*-gene hotspot mutations in canine fibrosarcomas and melanomas**

**Murua Escobar H, Günther K, Richter A, Soller JT, Winkler S, Nolte I  
and Bullerdiek J**

*Anticancer Res. (in press)*

Contribution to the work

- Planning and coordination of all done work
- *In silico* Analysis
- composing of the publication

## Absence of Ras-gene Hot-spot Mutations in Canine Fibrosarcomas and Melanomas

HUGO MURUA ESCOBAR<sup>1,2</sup>, KATHRIN GÜNTHER<sup>1</sup>, ANDREAS RICHTER<sup>1</sup>, JAN T. SOLLER<sup>1</sup>, SUSANNE WINKLER<sup>1</sup>, INGO NOLTE<sup>2</sup> and JÖRN BULLERDIEK<sup>1</sup>

<sup>1</sup>Centre for Human Genetics, University of Bremen, Leobener Strasse ZHG, 28359 Bremen;

<sup>2</sup>Small Animal Clinic, School of Veterinary Medicine, Bischofsholer Damm 15, 30173 Hanover, Germany

**Abstract.** Point mutations within *ras* proto-oncogenes, particularly within the mutational hot-spot codons 12, 13 and 61, are frequently detected in human malignancies and in different types of experimentally-induced tumours in animals. So far little is known about *ras* mutations in naturally occurring canine fibrosarcomas or *K-ras* mutations in canine melanomas. To elucidate whether *ras* mutations exist in these naturally occurring tumours in dogs, in the present study we screened 13 canine fibrosarcomas, 2 feline fibrosarcomas and 11 canine melanomas for point mutations, particularly within the mutational hot-spots, making this the first study to investigate a large number of canine fibrosarcomas. None of the samples showed a *K-* or *N-ras* hot spot mutation. Thus, our data strongly suggest that *ras* mutations at the hot-spot loci are very rare and do not play a major role in the pathogenesis of the spontaneously occurring canine tumours investigated.

Dogs and humans often share the same genetic pathways in the development of cancer, as has been described in the literature. Point mutations affecting genes of the *ras*- family are assumed to be among the most important alterations in human tumorigenesis (1). *Ras* proteins play an important role as signal transmitters. The binding of growth factors activates the *ras* protein and thus initiates cell division. Mutations in *ras* genes are assumed to lead to enduring activation of pathways that stimulate cell growth, which results in uncontrolled cell division (2). Especially mutations in *K-ras* have been described in human pancreatic cancers and tumours of the gastrointestinal tract, as well as in tumours of the skin (3-5). *K-ras* screening for hot-spot point mutations in dogs has been described in different types of lung cancer, pancreatic cancer

and breast cancer (6-12), showing that the canine gene is also affected by the typical *ras* mutations observed in humans but at a much lower ratio.

Guerrero *et al.* (13) were able to induce fibrosarcomas in nude mice by subcutaneously injecting transfected fibroblasts with *K-ras* point mutations affecting codon 12. So far little is known about *ras* mutations in canine fibrosarcomas. Just one report of a *ras* mutation screening including three canine fibrosarcoma samples has been described (11). There is also a lack of studies about *K-ras* mutations in canine melanomas are missing. In canine melanomas virtually no hot-spot *N-ras* mutations were described with one exception: Mayr *et al.* (14) found 2 out of 16 melanomas to be affected by mutations in codon 61.

In the present study, we screened 13 canine fibrosarcomas, 2 feline fibrosarcomas and 11 canine melanomas for point mutations, particularly within the mutational hot-spot codons of the *K-ras* and *N-ras* genes, to analyze whether these changes could be detected in these naturally occurring tumours.

### Materials and Methods

The tissues used in this study were provided by the Small Animal Clinic, School of Veterinary Medicine, Hanover, Germany. Thirteen canine fibrosarcoma, 2 feline fibrosarcoma and 11 canine melanoma samples were taken and used for analyses. The breeds represented were German Shorthaired Pointer, Irish Terrier, Fox Terrier, Schnauzer, Kuvasz, Berger de Brie, German Shepherd, Standard Poodle, Irish Red Setter, Rottweiler, Cairn Terrier, Beagle and canine and feline crossbreed.

The DNA of the twenty-six canine and feline fibrosarcoma and melanoma samples (10 - 25 mg each) was isolated using QIAamp DNA Kit (QIAGEN, Hilden, Germany) following the manufacturer's tissue protocol. The two feline samples served as internal controls, since they show specific point mutations compared to dogs (15). The PCRs for the screening of the hot-spot exons were performed using the following primer pairs. *K-ras*: primer pair KEx1up / KEx1lo (5' cgatataaggcctgctgaaa 3' / 5' ttaggatcatattcatcca 3') and primer pair KEx2up / KEx2lo (5' caggattctacaggaaca 3' / 5' aaccacataatggtgaa 3'). *N-ras*: primer pair NEx1up / NEx1lo (5' gactgagtaacaactggtg 3' / 5' gggcctcaccctatggtg 3') and primer pair NEx2up / NEx2lo (5' tcttaccgaaaacaggtggtatag 3' / 5' gtccctatgtattggtctctcatggcac3'). The PCR products were directly sequenced in the forward and reverse

Correspondence to: Dr. J. Bullerdiek, Centre for Human Genetics, University of Bremen, Leobener Strasse ZHG, 28359 Bremen, Germany. Tel: +49-421-218 4239, Fax: +49-421-218 4239, e-mail: bullerd@uni-bremen.de

Key Words: *Canis familiaris*, fibrosarcoma, hot-spot mutations, melanoma, *ras* genes.

Table I. Detected gene base substitutions in *N-ras* exon 1 and *K-ras* exons 1 and 2.

Gene /Exon	Sample	Codon	Substitution	Amino Acid Exchange
<i>K-ras</i> Exon 1	3	23	CTA→TTA	No AA exchange (Leu)
<i>K-ras</i> Exon 2	3	53	TTG→TAG	Leu→Stop
	13	70	CAG→CTG	Gln→Leu
	14	48	GGA→GAA	Gly→Glu
<i>N-ras</i> Exon 1	3	10	GGA→GAA	Gly→Glu
	24	22	CAG→CTG	Gln→Leu

direction and additionally cloned in pGEM-T Easy Vector System (Promega, Madison, USA) and sequenced once more. The DNA sequences and the homology alignments were created with various sequences from the NCBI database (accession numbers CFU62093, X02751, U62094, S42999, M54968, S64261). In case of single nucleotide exchanges being present, the procedures were repeated for verification.

## Results

Four of the twenty-six analysed samples showed nucleotide exchanges in the screened canine exons. None of the exchanges found affected the ras hot-spot codons 12, 13 and 61. One fibrosarcoma sample (Berger de Brie) showed three changes affecting *K-ras* exon 1 codon 23 (CTA→TTA, no amino acid exchange), exon 2 codon 53 (TTG→TAG, Leu→stop codon) and *N-ras* exon 1 codon 10 (GGA→GAA, Gly→Glu). Two other fibrosarcomas (Kuvasz and Poodle) each showed one nucleotide exchange in *K-ras* exon 2 affecting codon 48 (GGA→GAA, Gly→Glu) and codon 70 (CAG→CTG, Gln→Leu), respectively. *N-ras* exon 1 codon 22 (CAG→CTG, Gln→Leu) was affected in a melanoma sample (crossbreed) (Table I). The screening of *N-ras* exon 2 revealed no nucleotide exchanges among the canine sequences. The described nucleotide differences between the canine and feline sequences (15) in *N-ras* exon 2 and *K-ras* exon 2 were detected.

## Discussion

Our data strongly suggest that *K-* and *N-ras* mutations at the hot-spot loci are very rare and do not play a major role in the pathogenesis of the spontaneously occurring canine tumours investigated. These results are in accordance with the sparse data available for canine melanomas (twenty-four samples) and fibrosarcomas (three samples) (11, 14). In both studies a total of three mutations at the hot-spot codons could be detected. Compared to the data obtained from different studies in humans that show up to 30% of lung tumours, 90% of pancreatic tumours and 50% of tumours of the gastrointestinal tract to be affected by specific point mutations in the ras gene hot-spot codons (16, 17), the data seen in dogs apparently indicate that *ras* mutations do not play a major role in the pathogenesis of these spontaneously occurring canine tumours.

## References

- Arber N: Janus faces of ras: anti or pro-apoptotic? *Apoptosis* 4(5): 383-388, 1999.
- Park M: Genetic abnormalities of cell growth, *In: Scriver et al: The Metabolic and Molecular Basis of Inherited Disease*, 7 edn. New York: McGraw-Hill; 1995.
- Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N and Perucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* 53(4): 549-554, 1988.
- Belly RT, Rosenblatt JD, Steinmann M, Toner J, Sun J, Shehadi J, Peacock JL, Raubertas RF, Jani N and Ryan CK: Detection of mutated K12-ras in histologically negative lymph nodes as an indicator of poor prognosis in stage II colorectal cancer. *Clin Colorectal Cancer* 1(2): 110-116, 2001.
- Shukla VK, Hughes DC, Hughes LE, McCormick F and Padua RA: ras mutations in human melanotic lesions: K-ras activation is a frequent and early event in melanoma development. *Oncogene* Res 5(2): 121-127, 1989.
- Kraegel SA, Gumerlock PH, Dungworth DL, Oreffo VI and Madewell BR: K-ras activation in non-small cell lung cancer in the dog. *Cancer Res* 52(17): 4724-4727, 1992.
- Castagnaro M: [Ras gene analysis in mammary tumors of dogs by means of PCR-SSCP and direct genomic analysis]. *Ann Ist Super Sanita* 31(3): 337-341, 1995.
- Tierney LA, Hahn FF and Lechner JF: p53, erbB-2 and K-ras gene alterations are rare in spontaneous and plutonium-239-induced canine lung neoplasia. *Radiat Res* 145(2): 181-187, 1996.
- Griffey SM, Kraegel SA and Madewell BR: Rapid detection of K-ras gene mutations in canine lung cancer using single-strand conformational polymorphism analysis. *Carcinogenesis* 19(6): 959-963, 1998.
- Griffey SM, Kraegel SA, Weller RE, Watson CR and Madewell BR: K-ras mutations in 239PuO2 canine lung neoplasms. *Cancer Lett* 132(1-2): 1-5, 1998.
- Watzinger F, Mayr B, Gameraith R, Vetter C and Lion T: Comparative analysis of ras proto-oncogene mutations in selected mammalian tumors. *Mol Carcinog* 2001, 30(4):190-198.
- Mayr B, Schaffner G, Reifinger M: K-ras mutations in canine pancreatic cancers. *Vet Rec* 153(3): 87-89, 2003.
- Guerrero S, Figueras A, Casanova I, Farre L, Lloveras B, Capella G, Trias M and Mangués R: Codon 12 and codon 13 mutations at the K-ras gene induce different soft tissue sarcoma types in nude mice. *Faseb J* 16(12): 1642-1644, 2002.
- Mayr B, Schaffner G, Reifinger M, Zwetkoff S and Prodinger B: N-ras mutations in canine malignant melanomas. *Vet J* 165(2): 169-171, 2003.
- Watzinger F, Mayr B, Haring E and Lion T: High sequence similarity within ras exons 1 and 2 in different mammalian species and phylogenetic divergence of the ras gene family. *Mamm Genome* 9(3): 214-219, 1998.
- Knapp DW and Waters DJ: Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. *Mol Med Today* 3(1): 8-11, 1997.
- Bos JL: ras oncogenes in human cancer: a review. *Cancer Res* 49(17): 4682-4689, 1989.

Received March 22, 2004

Accepted June 14, 2004

## **II.**

### **Molecular characterization and mapping of the canine *Cyclin D1 (CCND1)* gene**

**Meyer B, Murua Escobar H, Winkler S, Richter A, Schelling C, Dolf G, Nolte I, and Bullerdiek J**

*Anim Genet. 2004; 35(5):413.*

Contribution to the work

- Planning and coordination of BAC screening
- Coordination of the cooperation
- Assistance at the composing of the publication

doi:10.1111/j.1365-2052.2004.01172.x

## Molecular characterization and mapping of the canine *cyclin D1* (*CCND1*) gene

B. Meyer\*, H. Murua Escobar<sup>\*,†</sup>, S. Winkler\*, G. Dolf<sup>‡</sup>, C. Schelling<sup>§</sup>, J. Bullerdiek\* and I. Nolte<sup>†</sup>

\*Center for Human Genetics, University of Bremen, Bremen, Germany. <sup>†</sup>Small Animal Clinic, School of Veterinary Medicine, Hanover, Germany. <sup>‡</sup>Institute of Animal Genetics, Nutrition and Housing, University of Berne, Berne, Switzerland. <sup>§</sup>Department of Animal Science, Swiss Federal Institute of Technology Zurich and Faculty of Veterinary Medicine, University of Zurich, Zurich, Switzerland

Accepted for publication 23 June 2004

**Introduction:** Cyclin D1, also known as PRAD1 or BCL-1, acts as regulator of progression through the G1 phase during the cell cycle by activation of cyclin-dependent kinases CDK4 and CDK6. In humans overexpression of cyclin D1, partially due to gene amplification, has been found in a wide variety of cancers, including breast cancer.<sup>1</sup>

**Sequence analysis:** For characterization of the canine *CCND1* gene and the corresponding protein, cDNA from a canine osteosarcoma was screened with primers specific for the ORF of human cyclin D1 (GenBank accession no. NM\_053056; primer pair CYCup: CGA TGC CAA CCT CCT CAA CGA, CYClo: TGT GGC ACA AGA GGC AAC GAA). After cloning and sequencing of the amplification product two additional primer sets were used to amplify the complete ORF (primer pairs Cyc1up: CAC ACG GAC TAC AGG GGA GT, Cyc333doglo: GCA CAC ACT TGA AGT AGG ACA C and Cyc695dogup: ACA CTT CCT CTC CAA GAT GCC, AP2: AAG GAT CCG TCG ACA TCT TTT TTT TTT TTT TTT T). Sequence analyses allowed the composition of a 1246 bp cDNA contig (GenBank accession no. AY620434), showing 90.4% sequence identity of the canine ORF compared with the human counterpart. In accordance with the human orthologue the deduced canine protein comprises 295 AA with 93.3% similarity between the two species.

**BAC library screening:** For use as FISH probe, a BAC clone was PCR-screened from the DogBAC library (<http://www.dogmap.ch>) with primers designed using human *CCND1* DNA sequence GenBank accession no. L09054 (primer pair CYCup: CGA TGC

CAA CCT CCT CAA CGA, CYCint1lo: GAA ACG TGG GTC TGG GCA ACA). The obtained positive BAC clone (DogBAC library ID S041P23D08) was verified by PCR, cloning and subsequent sequencing.

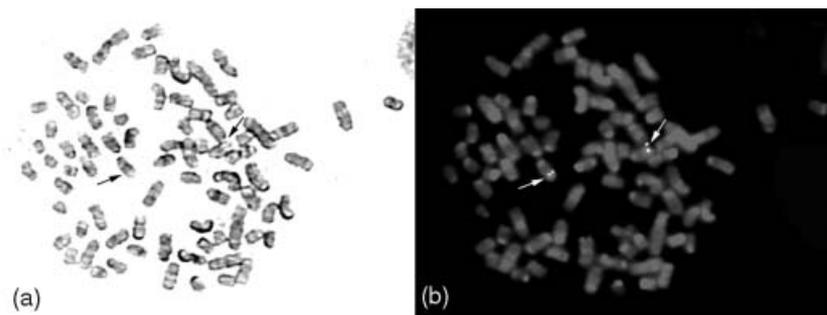
**Gene mapping:** For mapping of the chromosomal location of the canine *CCND1* gene, metaphase preparations and fluorescence *in situ* hybridization (FISH) were performed as described previously.<sup>2</sup> G-banded chromosomes were identified according to Reimann *et al.*<sup>3</sup> Ten well-spread metaphases were analysed exhibiting a signal on CFA17 on both chromatids of both chromosomes (Fig. 1).

**Comments:** During the last decade the dog has gained in importance as a model organism for the investigation of mechanisms underlying human genetic disease, including cancer. Immunohistochemical analyses of cyclin D1 expression in canine mammary tumours using a polyclonal antibody against human cyclin D1 revealed contradictory data. Murakami *et al.*<sup>4</sup> found cyclin D1 expression in only two adenocarcinomas of 75 mammary lesions tested whereas Sfacteria *et al.*<sup>5</sup> detected cyclin D1 in 60% of pre-cancerous lesions and 44% of cancerous lesions of the canine mammary gland with correlation of proliferative ratio and cyclin D1 expression. Mapping and sequencing of the canine *CCND1* gene and corresponding protein could help to elucidate the role of cyclin D1 in dogs and its usefulness as model organism concerning this matter. Yang *et al.*<sup>6</sup> found no conservation of synteny between HSA11, where the human *CCND1* maps, and CFA17. This discordance could be due to small rearrangements, deletions and insertions existing in the dog.<sup>7</sup>

### References

- Ormandy C. J. *et al.* (2003) *Breast Cancer Res Treat* **78**, 323–35.
- Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94**, 194–5.
- Reimann N. *et al.* (1996) *Cytogenet Cell Genet* **73**, 140–4.
- Murakami Y. *et al.* (2000) *J Vet Med Sci* **62**, 743–50.
- Sfacteria A. *et al.* (2003) *J Comp Pathol* **128**, 245–51.
- Yang F. *et al.* (1999) *Genomics* **62**, 189–202.
- Guyon R. *et al.* (2003) *Cold Spring Harb Symp Quant Biol* **68**, 171–8.

**Correspondence:** Prof. Dr Ingo Nolte (inolte@klt.tiho-hannover.de)



**Figure 1** Canine metaphase spread after GTG-banding (a) and the same metaphase after FISH with BAC S041P23D08 showing signals on both chromosomes 17 (b).

# **III.**

## **The FAS-activated serine/threonine kinase (*FASTK*) gene maps to canine chromosome 16**

**Meyer J, Murua Escobar H, Winkler S, Schelling C, Dolf G, Nolte I,  
and Bullerdiek J**

*Anim Genet. (in press)*

Contribution to the work

- Planning and coordination of BAC screening
- Coordination of the cooperations

# The FAS-activated serine/threonine kinase (FASTK) gene maps to canine chromosome 16

J. Meyer<sup>1,2</sup>, H. Murua Escobar<sup>1,2</sup>, S. Bartnitzke<sup>1</sup>, C. Schelling<sup>3</sup>, G. Dolf<sup>4</sup>,  
I. Nolte<sup>2</sup> and J. Bullerdiek<sup>1</sup>

<sup>1</sup> Center for Human Genetics, University of Bremen, Leobener Strasse ZHG,  
28359 Bremen, Germany

<sup>2</sup> Small Animal Clinic, School for Veterinary Medicine, Bischofsholer Damm 15,  
D-30173 Hannover, Germany

<sup>3</sup> Department of Animal Science, Swiss Federal Institute of Technology Zurich and  
Faculty of Veterinary Medicine, University of Zurich, 8092 Zurich, Switzerland

<sup>4</sup> Institute of Animal Genetics, Nutrition and Housing, University of Berne,  
Bremgartenstrasse 109a, 3012 Berne, Switzerland

Correspondence to:

Dr. J. Bullerdiek, Center for Human Genetics, University of Bremen,  
Leobener Strasse ZHG, D-28359 Bremen, Germany

Phone: +49-421-218-4239

Fax: +49-421-218-4239

E-mail: bullerd@uni-bremen.de

*Introduction:* FAS-activated serine/threonine kinase (*FASTK*) is activated by TNF- $\alpha$ , UV irradiation, heat shock and ceramide but not by mitogenic stimuli transmitted via the T-cell receptor complex. This observation indicates that *FASTK* is a stress-activated serine/threonine kinase involved in signalling apoptosis. *FASTK* is constitutively phosphorylated on serine and threonine residues and is activated by dephosphorylation. It is rapidly dephosphorylated and concomitantly activated to phosphorylate TIA-1 in response to FAS ligation. Activation of *FASTK* and phosphorylation of TIA-1 precede the onset of DNA fragmentation, suggesting that phosphorylated TIA-1 might signal downstream events in the apoptotic program.<sup>1</sup>

*FASTK*-mediated phosphorylation of TIA-1 plays a key role in apoptosis and regulates the translation of mRNAs encoding proteins essential for survival and/or proliferation.

The assignment of the canine *FASTK* gene was still unknown. In this study, we mapped the *FASTK* gene to canine chromosome (CFA) 16q14 by FISH.

*BAC clone and probe:* In order to generate a *FASTK* DNA probe, PCR amplification of genomic DNA from a two year old Golden Retriever was performed using primers that spanned part of exon 3 (primer up: GGT CAC CCT GAG CCC CAT GT and dn: GGT ACC CTC CCC GGT CCT GT).

PCR conditions: Total volume 50  $\mu$ l including 34,5  $\mu$ l Aqua Bidest, 5 $\mu$ l 10 x Buffer, 3  $\mu$ l MgCl<sub>2</sub>, 2  $\mu$ l dNTP's, 2  $\mu$ l of each Primer, approximat 50 ng genomic DNA and 0,5  $\mu$ l Taq-Polymerase. Thermocycler conditions: 10 min at 94°C, 35 cycles of 1 min 94°C, 1 min at 75°C, 2 min at 72°C and a final extension of 10 min at 72°C. The resulting amplicon of 420 bp was verified by sequencing. PCR conditions used to screen a canine BAC library<sup>2</sup> (URL: <http://www.dogmap.ch>) for *FASTK* positive

clones. To rule out false-positive BAC screening results, a PCR using the initial primer pair used for the screening probe was performed, cloned and sequenced for verification of BAC 24 D 10-1.

*Fluorescence in situ hybridization:* Metaphase preparations and fluorescence *in situ* hybridization (FISH) were performed as described previously<sup>3</sup>. Ten metaphases were examined and all demonstrated hybridization of the FASTK probe on both chromatids of CFA 16q14s.

*Comments:* It has been reported that the canine chromosome 16 at least shares homology with the human chromosomes (HSA) 4, 7 and 8. While the homologies with HSA 4, 7 are located at the long (q) arm of the chromosome the homologies with HSA 8 are shown to be distributed at the short (p) arm<sup>4</sup>. The human *FASTK* gene is located at HSA7q36.1. According to Yang et al.<sup>4</sup> exactly this region shares homology with CFA 16. We mapped the canine *FASTK* gene to CFA 16q14 following the nomenclature of Reimann et al<sup>5</sup>.

*References:*

- 1 Tian Q et al. (1995) *JexpMed* **182**, 865-74.
- 2 Schelling C, et al. (2002) *J Anim Breed Genet* **119**, 400-401
- 3 Murua Escobar H et al. (2001) *Cytogenet Cell Genet* **94**, 194-5.
- 4 Yang F et al. (1999) *Genomics* **62**, 189-202.
- 5 Reimann N et al. (1996) *Cytogenetics Cell Genetics* **73**, 140-144.

# IV.

## **The protein kinase B, gamma (*AKT3*) gene maps to canine chromosome 7**

**Murua Escobar H, Meyer J, Winkler S, Schelling C, Dolf G, Nolte I  
and Bullerdiek J**

*Anim Genet.* 2004; 35(4): 354-5.

Contribution to the work

- Establishment of the screening PCR
- Planning and coordination of BAC screening
- BAC DNA preparation
- Coordination of the international cooperation
- composing of the publication assisted by Janina Meyer

and 82% similarity to the above mentioned species, respectively.

**Chromosomal location:** Chromosomal localization was determined using a porcine-rodent somatic cell hybrid panel.<sup>4</sup> Primers CCS-RHAF and CCS-RHAR were designed to amplify an approximately 400 bp genomic fragment spanning part of exon 6, exon 7 and part of exon 8 of the porcine CCS. Statistical evaluation using the 'Interpreting PCR data' program (<http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm>) suggested a chromosome probability and correlation of 1.00 to the short arm of chromosome 2. The most likely localization for porcine CCS was 2p14-p17 with a probability of 0.7929 and a correlation of 0.8748. CCS is localized to human 11q13 and in mouse to the centromeric end of chromosome 19.<sup>5,6</sup> Human chromosome 11q13 shows conservation of synteny with the centromere of mouse chromosome 19 and porcine 2p14-17. The localization of markers flanking CCS is also conserved in pig and human, which supports the CCS mapping data and confirms that the gene described here is the pig orthologue of human CCS.

**PCR conditions:** For somatic cell hybridization – PCR was performed in 10 µl of reaction containing 10 ng DNA, 1X PCR buffer, 2.5 mM of each dNTP, 5 pmol of each primer, 3 µl 2% cresol red loading buffer and 0.5 U of *Taq* polymerase (Bioline, London, UK) under the following conditions: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s; 72 °C for 5 min.

**For cloning and sequencing** – PCR was performed using 3 µl cDNA (corresponding to 50 ng RNA) in a 15 µl reaction mixture containing 1X PCR buffer, 2.5 mM of each dNTP, 12 pmol of each primer and 0.1 U of *Taq* DNA polymerase (Amersham Biosciences, Hillerød, Denmark) in a thermal cycler (MJ Research, Waltham, MA, USA). The cycling conditions were 94 °C for 5 min; 40 cycles of 94 °C for 20 s, annealing temperature (indicated after primer sequences) for 20 s, 72 °C for 20 s; 72 °C for 10 min. Gel-purified amplification products were sequenced using Thermo Sequenase Terminator Cycle Sequencing kit (Amersham Life Science Inc.) in a thermal cycler (MJ research).

**Primers (5'→3'):** HS-CCS1244: CTCGGGGTGGTGACTG (55 °C)  
 HS-CCS1944: TCTGCTTGGGGTTCTGG (55 °C)  
 Pig-CCS2085: TAACCCTGATGGGATG (58 °C)  
 Poly-A 1576: AGCAGTGGTAACAACGCAGAGTACTTTTTTTT-  
 TTTTTTTTTT TTTTTTTTTTTT (58 °C)  
 CCS-RHAF: GGGGACCTAGGGAATGTCTGTG  
 CCS-RHAR: TCTGCTTGGGGTTCTGGAAGA

**Acknowledgements:** We gratefully acknowledge M. Yerle (INRA, Toulouse) for the pig-rodent panel. The Wilhelm Johannsen Centre for Functional Genome Research is established by the Danish National Research Foundation. A. N. Silaharoglu is supported by Danish Research Agency (project no: 2013-01-0033).

## References

- 1 Culotta V. C. *et al.* (1997) *J Biol Chem* **272**, 23469–72.
- 2 Rosen D. R. *et al.* (1993) *Nature* **362**, 59–62.

- 3 Silaharoglu A. N. *et al.* (2002) *BMC Genet* **19**, 5.
- 4 Yerle M. *et al.* (1996) *Cytogenet Cell Genet* **73**, 194–202.
- 5 Bartnikas T. B. *et al.* (2000) *Mamm Genome* **11**, 409–11.
- 6 Moore S. D. P. *et al.* (2000) *Cytogenet Cell Genet* **88**, 35–7.

**Correspondence:** Asli N. Silaharoglu MSc., PhD  
 (asli@medgen.ku.dk)

doi:10.1111/j.1365-2052.2004.01153.x

## The protein kinase B, gamma (*AKT3*) gene maps to canine chromosome 7

**H. Murua Escobar<sup>\*,†</sup>, J. Meyer<sup>\*,†</sup>, S. Winkler<sup>\*</sup>,  
 C. Schelling<sup>‡</sup>, G. Dolf<sup>§</sup>, I. Nolte<sup>†</sup> and J. Bullerdiek<sup>\*</sup>**

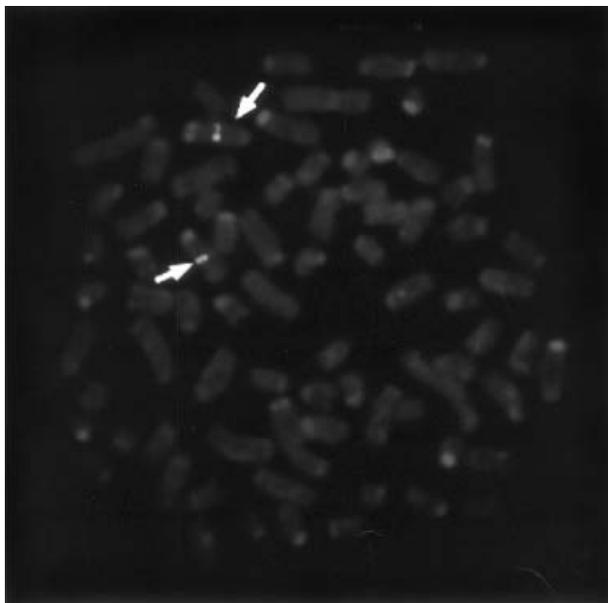
<sup>\*</sup>Center for Human Genetics, University of Bremen, Leobener Strasse ZHG, 28359 Bremen, Germany. <sup>†</sup>Small Animal Clinic, School for Veterinary Medicine, Bischofsholer Damm 15, D-30173 Hannover, Germany. <sup>‡</sup>Department of Animal Science, Swiss Federal Institute of Technology Zurich and Faculty of Veterinary Medicine, University of Zurich, 8092 Zurich, Switzerland. <sup>§</sup>Institute of Animal Genetics, Nutrition and Housing, University of Berne, Bremgartenstrasse 109a, 3012 Berne, Switzerland

Accepted for publication 19 April 2004

**Introduction:** The protein kinase B, gamma (*AKT3*) protein is an intracellular serine/threonine kinase involved in regulating cell survival. This protein phosphorylates and regulates the function of many cellular proteins involved in processes that include metabolism, apoptosis and proliferation,<sup>1,2</sup> making it a promising target for drug discovery to treat cancer. Expression of the human gene is found in normal and tumour tissues. Prior to this study, the assignment of the canine *AKT3* gene was unknown. Herein, we report the assignment of the *AKT3* gene to canine chromosome (CFA) 7q17 by FISH.

**BAC clone and probe:** In order to generate an *AKT3* DNA probe, polymerase chain reaction (PCR) amplification of genomic DNA from a 2-year-old Golden retriever was performed using primers that spanned part of exon 13 (primer up: AGA CAG TAG CAG CAG CAG CA and dn: ATG ACG AGG ACG GTA TGG AC). Primers were designed using NCBI Sequence AY575066, which shows 80.3% identity to human *AKT3* mRNA (NM\_005465).

**PCR conditions:** The total volume of 50 µl included 34.5 µl Aqua Bideest, 1x Buffer, 3 mM MgCl<sub>2</sub>, 2 mM dNTPs, 2 µM of each primer, approximately 50 ng genomic DNA and 2.5 U *Taq*-Polymerase. Thermocycler conditions were as follows: 10 min at 94 °C, 35 cycles of 1 min 94 °C, 1 min at 75 °C, 2 min at 72 °C and a final extension of 10 min at 72 °C. The resulting amplicon of 303 bp was verified by sequencing (GenBank accession no. AY575065). These PCR primers and conditions were also used to screen a canine BAC library<sup>3</sup> (URL: <http://www.dogmap.ch>) for *AKT3* positive clones. To rule out false-positive BAC screening results, the initial PCR was repeated,



**Figure 1** Metaphase spread after fluorescence *in situ* hybridization with signals on both chromosome 7s (above) and GTG-banding (below).

cloned and sequenced for verification of *AKT3* with BAC clone 10C05-4.

**Fluorescence *in situ* hybridization:** Metaphase preparations and fluorescence *in situ* hybridization (FISH) were performed as described previously.<sup>4</sup> Ten metaphases were examined and all demonstrated hybridization of the *AKT3* probe on both chromatids of canine chromosome 7 (Fig. 1).

**Comments:** It has been reported that canine chromosome 7 shares homology with human chromosomes (HSA) 1 and 18. The long (q) arm of CFA7 corresponds to the homologous region on HSA1, whereas the homologous regions with HSA18 are distributed over both arms of CFA7.<sup>5</sup> The human *AKT3* gene is located at HSA 1q43–44. According to Yang *et al.*,<sup>5</sup> this region shares homology with CFA7. We mapped the

canine *AKT3* gene to CFA 7q17 and defined the chromosomal band following the nomenclature established by Reimann *et al.*<sup>6</sup>

## References

- 1 Masure S. *et al.* (1999) *Eur J Biochem* **265**, 353–60.
- 2 Nicholson K. M. *et al.* (2002) *Cell Signal* **14**, 381–95.
- 3 Schelling C. *et al.* (2002) *J Anim Breed Genet* **119**, 400–1.
- 4 Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94**, 194–5.
- 5 Yang F. *et al.* (1999) *Genomics* **62**, 189–202.
- 6 Reimann N. *et al.* (1996) *Cytogenetics Cell Genetics* **73**, 140–4.

Correspondence: J. Bullerdiek (bullerd@uni-bremen.de)

doi:10.1111/j.1365-2052.2004.01158.x

## The canine *NRAS* gene maps to CFA 17

**A. Richter\***, **H. Murua Escobar\*<sup>†</sup>**, **K. Günther\***, **B. Meyer\***, **S. Winkler\***, **G. Dolf<sup>‡</sup>**, **C. Schelling<sup>§</sup>**, **I. Nolte<sup>†</sup>** and **J. Bullerdiek\***

\*Centre for Human Genetics, University of Bremen, Bremen, Germany. <sup>†</sup>Small Animal Clinic, School of Veterinary Medicine Hanover, Hanover, Germany. <sup>‡</sup>Institute of Animal Genetics, Nutrition and Housing, University of Berne, Berne, Switzerland. <sup>§</sup>Department of Animal Science, Swiss Federal Institute of Technology Zurich, Faculty of Veterinary Medicine, University of Zurich, ETH-Zentrum, Zurich, Switzerland

Accepted for publication 1 May 2004

**Introduction:** The dog is an emerging model organism for the investigation of mechanisms involved in human disease, including cancer. Several parallels in human and canine tumours have been described, with comparable environmental living conditions and age of tumour onset in both human and canine patients as well as similarities in development and histology of tumours in both species.<sup>1</sup> *NRAS* is a member of the *ras* proto-oncogene family of proteins that act in growth-related signal transduction and are frequently involved in the development of human tumours, with *ras* point mutations being one of the most important alterations in the onset of malignancies.<sup>2</sup> *Ras* genes show high sequence similarity across different mammalian species such as human, cat, dog, cattle and rodents, with most nucleotide differences representing synonymous changes not affecting the amino acid sequence.<sup>3</sup> In malignancies, most amino acid exchanges in *ras* genes are caused by alterations of the so-called hot spot codons 12, 13, and 61 in exons 1 and 2, respectively, leading to constitutively active *ras* proteins that bring about constant signal transduction, facilitating uncontrolled cell division. These hot-spot codons have been described to be affected in other mammalian species as well. In dogs, *NRAS* mutations were found in lymphomas<sup>4</sup> and malignant melanomas.<sup>5</sup>

The canine *NRAS* gene had not been mapped so far, therefore, in this study we localized the chromosomal location of the canine *NRAS* gene by fluorescence *in situ* hybridization (FISH).

# V.

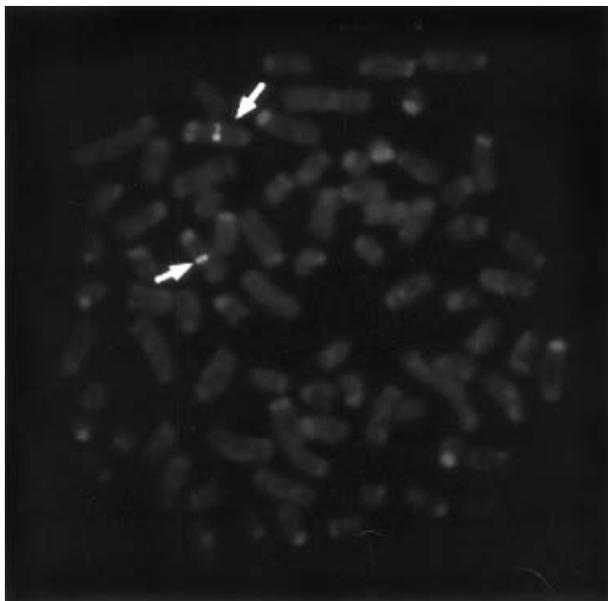
## **The canine *NRAS* gene maps to CFA 17**

**Richter A, Murua Escobar H, Günther K, Meyer B, Winkler S, Dolf G,  
Schelling C, Nolte I and Bullerdiek J**

*Anim Genet.* 2004; 35(4): 355-6.

Contribution to the work

- Establishment of the screening PCR
- Planning and coordination of BAC screening
- Coordination of the international cooperation
- Assistance at the composing of the publication



**Figure 1** Metaphase spread after fluorescence *in situ* hybridization with signals on both chromosome 7s (above) and GTG-banding (below).

cloned and sequenced for verification of *AKT3* with BAC clone 10C05-4.

**Fluorescence *in situ* hybridization:** Metaphase preparations and fluorescence *in situ* hybridization (FISH) were performed as described previously.<sup>4</sup> Ten metaphases were examined and all demonstrated hybridization of the *AKT3* probe on both chromatids of canine chromosome 7 (Fig. 1).

**Comments:** It has been reported that canine chromosome 7 shares homology with human chromosomes (HSA) 1 and 18. The long (q) arm of CFA7 corresponds to the homologous region on HSA1, whereas the homologous regions with HSA18 are distributed over both arms of CFA7.<sup>5</sup> The human *AKT3* gene is located at HSA 1q43–44. According to Yang *et al.*,<sup>5</sup> this region shares homology with CFA7. We mapped the

canine *AKT3* gene to CFA 7q17 and defined the chromosomal band following the nomenclature established by Reimann *et al.*<sup>6</sup>

## References

- 1 Masure S. *et al.* (1999) *Eur J Biochem* **265**, 353–60.
- 2 Nicholson K. M. *et al.* (2002) *Cell Signal* **14**, 381–95.
- 3 Schelling C. *et al.* (2002) *J Anim Breed Genet* **119**, 400–1.
- 4 Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94**, 194–5.
- 5 Yang F. *et al.* (1999) *Genomics* **62**, 189–202.
- 6 Reimann N. *et al.* (1996) *Cytogenetics Cell Genetics* **73**, 140–4.

Correspondence: J. Bullerdiek (bullerd@uni-bremen.de)

doi:10.1111/j.1365-2052.2004.01158.x

## The canine *NRAS* gene maps to CFA 17

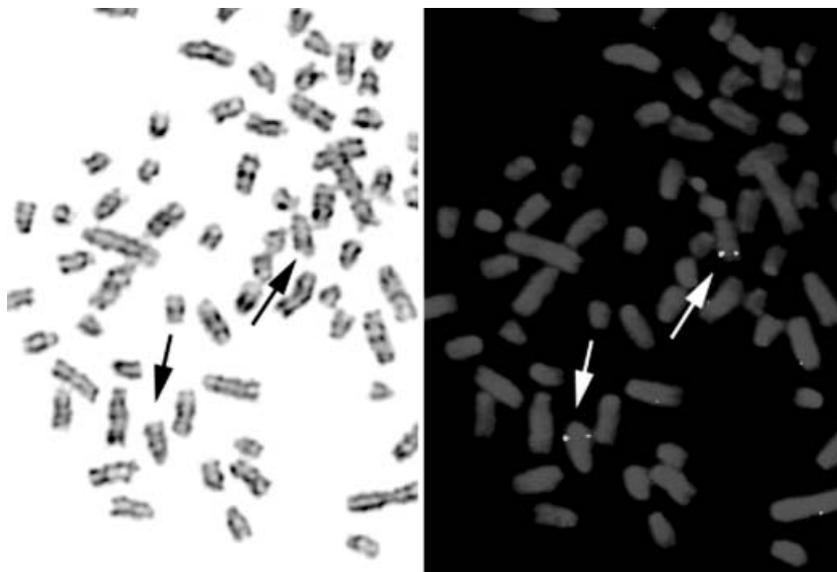
**A. Richter\***, **H. Murua Escobar\*<sup>†</sup>**, **K. Günther\***, **B. Meyer\***, **S. Winkler\***, **G. Dolf<sup>‡</sup>**, **C. Schelling<sup>§</sup>**, **I. Nolte<sup>†</sup>** and **J. Bullerdiek\***

\*Centre for Human Genetics, University of Bremen, Bremen, Germany. <sup>†</sup>Small Animal Clinic, School of Veterinary Medicine Hanover, Hanover, Germany. <sup>‡</sup>Institute of Animal Genetics, Nutrition and Housing, University of Berne, Berne, Switzerland. <sup>§</sup>Department of Animal Science, Swiss Federal Institute of Technology Zurich, Faculty of Veterinary Medicine, University of Zurich, ETH-Zentrum, Zurich, Switzerland

Accepted for publication 1 May 2004

**Introduction:** The dog is an emerging model organism for the investigation of mechanisms involved in human disease, including cancer. Several parallels in human and canine tumours have been described, with comparable environmental living conditions and age of tumour onset in both human and canine patients as well as similarities in development and histology of tumours in both species.<sup>1</sup> *NRAS* is a member of the *ras* proto-oncogene family of proteins that act in growth-related signal transduction and are frequently involved in the development of human tumours, with *ras* point mutations being one of the most important alterations in the onset of malignancies.<sup>2</sup> *Ras* genes show high sequence similarity across different mammalian species such as human, cat, dog, cattle and rodents, with most nucleotide differences representing synonymous changes not affecting the amino acid sequence.<sup>3</sup> In malignancies, most amino acid exchanges in *ras* genes are caused by alterations of the so-called hot spot codons 12, 13, and 61 in exons 1 and 2, respectively, leading to constitutively active *ras* proteins that bring about constant signal transduction, facilitating uncontrolled cell division. These hot-spot codons have been described to be affected in other mammalian species as well. In dogs, *NRAS* mutations were found in lymphomas<sup>4</sup> and malignant melanomas.<sup>5</sup>

The canine *NRAS* gene had not been mapped so far, therefore, in this study we localized the chromosomal location of the canine *NRAS* gene by fluorescence *in situ* hybridization (FISH).



**Figure 1** Metaphase spread after fluorescence *in situ* hybridization showing signals on both chromosomes 17 (right) and the same metaphase after GTG-banding (left).

**BAC library screening:** In order to isolate a FISH probe, the DogBAC canine BAC library<sup>6</sup> (<http://www.dogmap.ch/>) was polymerase chain reaction (PCR)-screened. Primers were designed using canine mRNA sequence GenBank accession no. U62093 (primer UP: GACTGAGTACAAACTGGTGG and primer LO: GGGCCTCACCTCTATGGTG). The PCR conditions were established on canine blood genomic DNA, the corresponding PCR product cloned and verified by sequencing. The positive BAC clone (DogBAC library ID S050P24H09) was verified by PCR and sequencing.

**Gene mapping:** For mapping the chromosomal location of the canine *NRAS* gene, metaphase preparations and FISH were performed as described previously.<sup>7</sup> Ten well spread metaphases exhibited a signal on CFA 17 on both chromatids of both chromosomes (Fig. 1), following the nomenclature of the canine karyotype as established by Reimann *et al.*<sup>8</sup>

**Comments:** *NRAS* mutations in humans have been found in 30% of liver tumours, 40% of myelodysplastic syndrome, 30% of acute myelogenous leukaemia, 13% of brain tumours and in 53% of follicular and 60% of undifferentiated papillary thyroid tumours.<sup>9</sup> In dogs, depending on tumour type, comparable occurrences exist in malignant melanomas,<sup>5</sup> while fibrosarcomas showed no amino acid alteration of the *NRAS* protein (H. Murua Escobar, K. Günther, A. Richter, J. T. Soller, S. Winkler, I. Nolte & J. Bullerdiek 2004, personal communication). Overall, data available on involvement of *ras* proto-oncogenes in tumours of dogs are still insufficient. Knowledge of the cytogenetic properties of *NRAS* will further the understanding of this important gene. The mapping results obtained in this study are in accordance with the known homology between canine chromosome 17 and the centromer-proximal regions 11.1–13.3 of the p-arm of human chromosome 1.<sup>10</sup>

## References

- Hahn K A. *et al.* (1994) *In Vivo* **8**, 133–43.
- Arber N. (1999) *Apoptosis* **4**, 383–8.
- Watzinger F. *et al.* (1998) *Mamm Genome* **9**, 214–9.
- Mayr B. *et al.* (2003) *Acta Vet Hung* **51**, 91–4.
- Mayr B. *et al.* (2003) *Vet J* **165**, 169–71.
- Schelling C. *et al.* (2002) *J Anim Breeding Genet* **119**, 400–1.
- Becker K. *et al.* (2003) *Anim Genet* **34**, 68–9.
- Reimann N. *et al.* (1996) *Cytogenet Cell Genet* **73**, 140–4.
- Spandidos D. A. *et al.* (2002) *Int J Oncol* **21**, 237–41.
- Yang F. *et al.* (1999) *Genomics* **62**, 189–202.

Correspondence: J. Bullerdiek (bullerd@uni-bremen.de)

doi:10.1111/j.1365-2052.2004.01159.x

## Linkage mapping of chicken *ovoinhibitor* and *ovomuroid* genes to chromosome 13

**K. Kinoshita\***, **T. Shimogiri<sup>†</sup>**, **S. Okamoto<sup>†</sup>**, **K. Yoshizawa<sup>‡</sup>**, **H. Mannen<sup>‡</sup>**, **H. R. Ibrahim<sup>†</sup>**, **H. H Cheng<sup>§</sup>** and **Y. Maeda<sup>†</sup>**

\*The United Graduate School of Agricultural Sciences, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan. <sup>†</sup>Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan. <sup>‡</sup>Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657-8501, Japan. <sup>§</sup>United States Department of Agriculture, Agriculture Research Service, East Lansing, MI 48823, USA

Accepted for publication 10 May 2004

**Source/description:** Ovoinhibitor (OIH) and ovomucoid (OVM) are the major proteinase inhibitors constituting 1.5 and 11% of the total proteins in hen egg white, respectively. Although OVM exerts its antiprotease activity only against trypsin, OIH has a wide spectrum of inhibitory activity for other proteinases that occur in chicken egg white and blood plasma.<sup>1</sup> They are functionally similar proteins and having multiple domains with a characteristic pattern of disulphide bridges.<sup>1</sup> From the analysis of DNA sequences and the positions of exons and introns, it

# VI.

## The canine *KRAS2* gene maps to CFA 22

**Winkler S, Murua Escobar H, Günther K, Richter A, Dolf G, Schelling C, Bullerdiek J Nolte I**

*Anim Genet. 2004; 35(4): 350-1.*

Contribution to the work

- Establishment of the screening PCR
- Planning and coordination of BAC screening
- BAC DNA preparation
- Coordination of the international cooperation
- Assistance at the composing of the publication

## The canine *KRAS2* gene maps to chromosome 22

S. Winkler\*, H. Murua Escobar<sup>\*,†</sup>, K. Günther\*, A. Richter\*, G. Dolf<sup>‡</sup>, C. Schelling<sup>§</sup>, J. Bullerdiek\* and Ingo Nolte<sup>†</sup>

\*Center for Human Genetics, University of Bremen, Bremen, Germany. <sup>†</sup>Small Animal Clinic, School of Veterinary Medicine, Hannover, Germany. <sup>‡</sup>Institute of Animal Genetics, Nutrition and Housing, University of Berne, Berne, Switzerland. <sup>§</sup>Department of Animal Science, Swiss Federal Institute of Technology Zürich and Faculty of Veterinary Medicine, University of Zürich, Zürich, Switzerland

Accepted for publication 20 March 2004

**Introduction:** Dogs and human beings often share the same genetic pathways in development of cancer. Point mutations affecting genes of the *ras* family are assumed to be among the most important alterations in human tumorigenesis.<sup>1</sup> Ras proteins play an important role as signal transmitters. The binding of growth factors activate the ras protein and thus initiates cell division. Mutations in *ras* genes are assumed to remove the time limit of the cell stimulating signals which results in uncontrolled cell division.<sup>2</sup> Mutations in *KRAS2* have been described in human pancreatic cancers and tumours of the gastrointestinal tract as well as in tumours of the skin.<sup>3–5</sup> Hot spot point mutations in *KRAS2* described in different types of human lung tumours and breast cancers are also present in the corresponding canine gene.<sup>6</sup> For further characterization of the gene, we have mapped the canine *KRAS2* gene.

**BAC clone and probe:** A *KRAS2* DNA probe was generated by polymerase chain reaction (PCR) spanning part of the exon 2 (primer up: 5'-caggattctacaggaaca-3'/lo: 5'-aacccacctataatggtgaa-3' based on NCBI sequence M54968) using genomic canine DNA. The resulting amplicon was cloned and sequenced for verification. These PCR conditions were also used to screen a canine BAC library<sup>7</sup> (URL: <http://www.dogmap.ch>). To rule out false-positive BAC screening results, a PCR using the initial primer pair was performed,

and the resulting amplicon cloned and sequenced for verification. BAC S069P22D02 was positive for *KRAS2* and was used for fluorescence *in situ* hybridization (FISH) analysis.

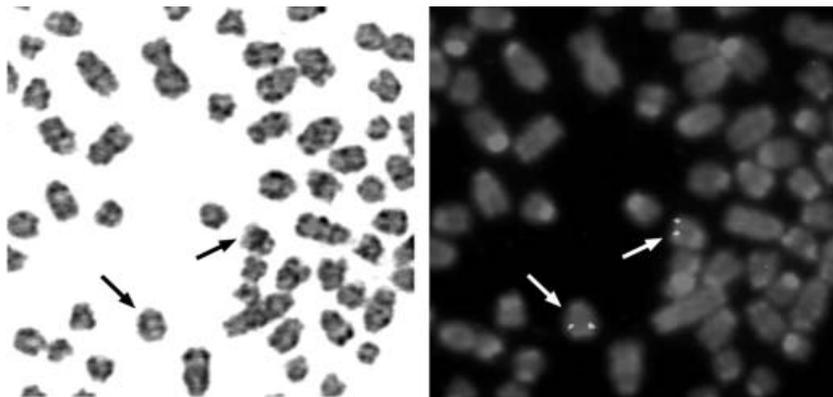
**FISH:** Metaphase preparations and FISH were performed as described previously.<sup>8</sup> Ten well spread metaphases were examined and all showed a signal on both chromatids of chromosome 22s (CFA 22) (Fig. 1).

**Comments:** Different investigations show that 30% of human lung tumours, 90% of human pancreatic tumours and 50% of tumours of the gastrointestinal tract depend on specific point mutations in genes of the *ras* gene family.<sup>9–11</sup> Molecular investigations of the *ras* family are rare in dogs, but existing publications point to the fact that there are the same point mutations affecting hotspot codons 12, 13 and 61, as they are in human malignancies.<sup>6</sup> Up to 24% of cases investigated in dogs showed point mutations in those codons. The canine chromosome to which *KRAS2* was mapped has been involved in a centric fusion of CFA 8/22.<sup>12</sup> According to Yang *et al.*<sup>13</sup> the canine chromosome 22 shares homology with HSA13, whereas the human *KRAS2* gene is located on HSA12. In our FISH studies, there were no metaphase signals on the corresponding canine chromosomes, 29 and 10.

**Acknowledgements:** We thank Norbert Drieschner for his helpful advice in FISH techniques.

### References

- Arber N. (1999) *Apoptosis* 4, 383–8.
- Scriver *et al.* eds (1995) *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, 7th edn, pp. 589–611.
- Almoguera C. *et al.* (1988) *Cell* 53, 549–54.
- Belly R. T. *et al.* (2001) *Clin Colorectal Cancer* 1, 110–6.
- Shukla V. K. *et al.* (1989) *Oncogene Res* 5, 121–7.
- Watzinger F. *et al.* (2001) *Mol Carcinog* 30, 190–8.
- Schelling C. *et al.* (2002) *J Anim Breeding Genet* 119, 400–1.
- Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* 94, M194–5.
- Bos J. L. *et al.* (1989) *Cancer Res* 50, 1352.
- Tang E. M. *et al.* (2002) *J Natl Cancer Inst* 94, 1527–36.



**Figure 1** Metaphase spread after fluorescence *in situ* hybridization with signals on both chromosomes 22 (right) and the same metaphase after GTG banding (left).

- 11 Knapp D. W. & Waters D. J. (1997) *Mol Med Today* 3, 8–11.  
 12 Mayr B. *et al.* (1991) *Br Vet J* 6, 545–8.  
 13 Yang F. *et al.* (1999) *Genomics* 62, 189–202.

Correspondence: Prof. Dr Jörn Bullerdiek (bullerd@uni-bremen.de)

doi:10.1111/j.1365-2052.2004.01148.x

## Genomic localization and SNP discovery in the bovine melanocortin receptor 4 gene (*MC4R*)

E. Valle\*, F. A. Habermann<sup>†</sup>, S. S. Moore<sup>‡</sup>,  
 D. H. Crews\* and B. F. Benkel\*

\*Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, Alberta, Canada. <sup>†</sup>Chair of Animal Breeding, Technical University of Munich, Freising-Weißenstephan, Germany.

<sup>‡</sup>Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada

Accepted for publication 12 April 2004

**Source/description:** *MC4R* is a G-protein-coupled receptor that is implicated in the control of food intake and energy expenditure. In mice, knock out of the *Mc4r* gene results in a maturity-onset obesity syndrome which is due primarily to leptin-resistant hyperphagia.<sup>1,2</sup> Moreover, a significant association has been reported between *MC4R* genotypes and backfat, growth rates, and feed intake in a number of lines of swine.<sup>3</sup> *MC4R* is an intron-less gene with a transcript of 1800 nt containing a coding region of roughly 1 kb. *MC4R* has been assigned to the telomeric region of BTA24 by radiation hybrid mapping<sup>4</sup> (RH) and linkage analysis.<sup>5</sup> Both papers also report SNPs in the *MC4R* coding region – two single nucleotide substitutions specific to the Red Holstein and Red Pied breeds,<sup>4</sup> and a single nucleotide substitution in cattle of unspecified origin.<sup>5</sup>

In this study we report: (i) the anchoring of the gene to a specific contig in the bovine reference BAC genome scaffold; (ii) the cytogenetic localization of the bovine *MC4R* gene using FISH; and (iii) the results of an SNP discovery experiment using a beef cattle reference panel.

### Primer sequences:

#### Overgo hybridization

MC4RP1: 5'-GCCTAAGATTTCCAAGTGATGCT

MC4RP2: 5'-AAGTGTGGCTCTGGTCAGCATCAC

#### PCR verification

MC4RRev: 5'-AAAGTTAGCGCGGAGAGA

#### SNP discovery

Forward: 5'-GATTTCCAAGTGATGCTGACC

Reverse: 5'-ACACACAGTATGGGTTCTGGG

**Overgo hybridization to BAC filters:** High density filters for the bovine BAC library CHORI-240 were purchased from the BAC-PAC Resource Centre (<http://bacpac.chori.org/home.htm>). Overgo probe oligomers (MC4RP1 and MC4RP2) and the PCR verification primer (MC4RRev) were designed using the Overgo 1.02 program (<http://www.mouse-genome.bcm.tmc.edu/web/overgo/OvergoInput.asp>). Overgo probes were labelled and hybridizations to high density filters carried out as previously described.<sup>6</sup> Hybridization-positive BAC clones were confirmed via locus-specific PCR reactions using primers MC4RP1 and MC4RRev revealing three clones for the *MC4R* gene – CHORI-240 180D6, 213B5 and 265F11. All three clones map to contig no. 926 in the bovine reference BAC genome scaffold (<http://www.bcgsc.ca/lab/mapping/bovine>), thereby anchoring this contig of 95 BACs to the telomeric end of BTA24.

**Fluorescent in situ hybridization (FISH):** BAC DNA for clone 180D6 was prepared using the R.E.A.L. Prep BAC kit (Qiagen, Mississauga, ON, Canada). BAC DNA was labelled with digoxigenin-11-dUTP by standard nick translation and hybridized with a 10× excess of bovine Cot1-DNA to normal male bovine metaphase spreads. Probe hybridization was detected with monoclonal mouse-anti digoxigenin (Roche, Mannheim,

**Table 1** SNP discovery in the bovine *MC4R* gene.

Nucleotide position <sup>1,2</sup>	244	270	271	414	648	747	927
Codon	11	19	20	67	145	178	238
<i>Bos taurus</i>							
Angus <sup>3</sup>	A	C	G	G	G	G	C
Charolais	A	C	G	G	G	G	C
Hereford	A	C	G	G	G	G	C
Limousin	A	C	G	G	G	4G/2A (silent) <sup>4</sup>	5C/1T (silent)
Simmental	A	C	G	G	G	G	C
Holstein	A	C	G	G	G	G	C
Wagyu	A	C	G	G	G	G	C
<i>Bos indicus</i>							
Brahman	A	C	G	G	G	G	C
<i>Bison bison</i>							
	G (T11A)	5C/1T (silent)	1G/5A (A20T)	5G/1A (silent)	1G/5A (silent)	G	C

<sup>1</sup>Positions are numbered according to GenBank accession no. AF265221.

<sup>2</sup>SNPs detected previously at positions 647 and 727<sup>4</sup> were not observed in this study, and the previously reported SNP at position 1069<sup>5</sup> lies downstream of the fragment analysed.

<sup>3</sup>Three unrelated bulls (six alleles) were sampled from each breed.

<sup>4</sup>The impact of the SNP on the amino acid at the affected position is indicated in brackets.

# VII.

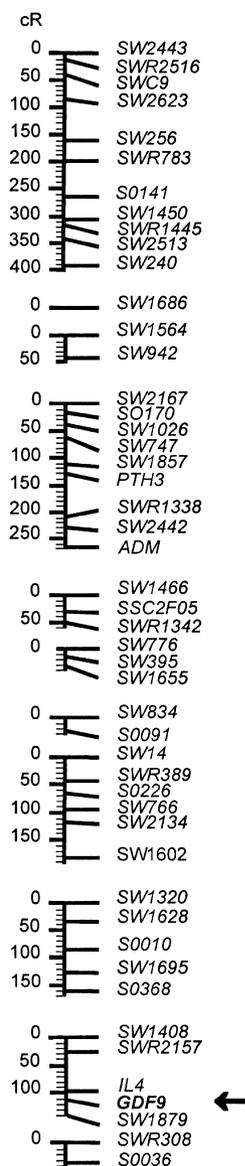
## **Molecular characterization and mapping of the canine KRAB zinc finger gene *ZNF331***

**Meiboom M, Murua Escobar H, Winkler S, Nolte I, Bullerdiek J**

*Anim Genet. 2004; 35(3): 262-3.*

Contribution to the work

- Partial cloning of the CDS
- Construction of the cDNA Library
- Planning and coordination of all phage work
- Radioactive screening of the BAC incl. planning and coordination



**Figure 2** Radiation hybrid map of porcine chromosome 2<sup>B</sup> showing position of *GDF9* gene.

524/01/0903). We thank Mrs Markéta Hančová for technical assistance.

## References

- 1 Bodensteiner K. J. *et al.* (1999) *Biol Reprod* **60**, 381–6.
- 2 Committee for Standardized Karyotype of the Domestic Pig (1988) *Hereditas* **109**, 151–7.
- 3 Trask B. J. (1991) *Meth Cell Biol* **35**, 3–35.
- 4 NCBI database, Gene ID: 2661 (URL = <http://www.ncbi.nlm.nih.gov/LocusLink/LocRptcgi?l=2661>).
- 5 Goureau A. *et al.* (1996) *Genomics* **36**, 252–62.
- 6 Yerle M. *et al.* (1998) *Cytogenet Cell Genet* **82**, 182–8.
- 7 Milan D. *et al.* (2000) *Bioinformatics* **16**, 558–9.
- 8 Hawken R. J. *et al.* (1999) *Mamm Genome* **10**, 824–30.
- 9 Dong J. *et al.* (1996) *Nature* **383**, 531–5.

10 McNatty K. P. *et al.* (2003) *Reprod Suppl* **61**, 339–51.

11 Bidanel J. P. & Rothschild M. (2002) *Pig News Info* **23**, 39N–54N.

Correspondence: S. Čepica ([cepica@iapg.cas.cz](mailto:cepica@iapg.cas.cz))

doi:10.1111/j.1365-2052.2004.01146.x

## Molecular characterization and mapping of the canine KRAB zinc finger gene *ZNF331*

**M. Meiboom\***, **H. Murua Escobar<sup>†,\*</sup>**, **S. Winkler\***, **I. Nolte<sup>†</sup>** and **J. Bullerdiek\***

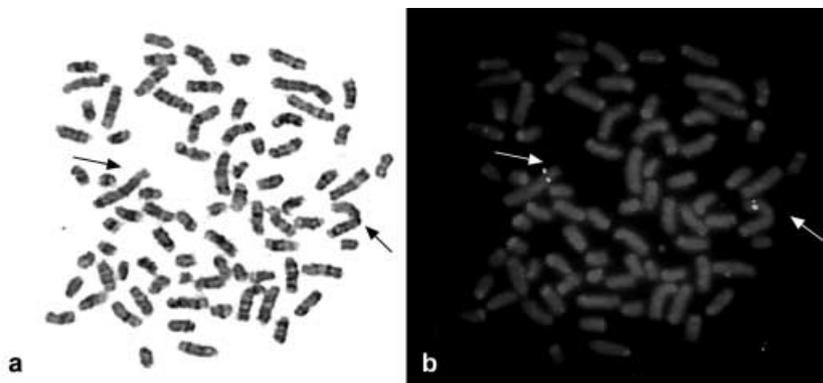
\*Center for Human Genetics, University of Bremen, Bremen, Germany. <sup>†</sup>Small Animal Clinic, Hannover School of Veterinary Medicine, Hannover, Germany

Accepted for publication 12 April 2004

**Source/description:** *ZNF331* is a KRAB zinc finger protein gene consisting of a KRAB-A box and a zinc finger domain with 12 zinc fingers and has recently been identified as putative target gene in thyroid tumorigenesis.<sup>1,2</sup> For characterization of canine *ZNF331*, a canine testis cDNA library (Center for Human Genetics, Bremen, Germany) was screened with primers specific for human *ZNF331* (acc.-no. NM\_018555; Primer Up: GTA AAT CCC TTG GCC GTA ACT G; Lo: AGG CCT TCC CAC ATT CTT GAC). To obtain a full-length cDNA clone 5'RACE-PCRs with a vector-specific primer [Primer Up: AGC GGA TAA CAA TTT CAC ACA GG (M13rev)] and a gene-specific primer (Primer Lo: TAT TTT CTC TAC AAG TGG GCG TTT T) were performed using the cDNA library as template. Sequence analysis of the isolated clone and the 5' RACE products allowed the assembling of the mRNA sequence of *ZNF331*. The composed canine *ZNF331* cDNA (GenBank acc. no. AY375188) consists of 2148 bp including the full ORF and shows 85.3% sequence identity in the cds to the human *ZNF331* gene.

Expression studies using Northern blots containing mRNA from several canine tissues including testis and a canine *ZNF331* spacer-specific probe did not reveal transcripts of canine *ZNF331* which points to a very low expression level of this gene (data not shown).

**Fluorescent in situ hybridization:** A cDNA probe representing the spacer region of canine *ZNF331* was used to screen the canine RCPI 81 BAC/PAC filter (BACPAC RESOURCES, Childrens Hospital, Oakland, CA, USA). The probe was generated by *EcoRI* digestion of a PCR product of primers CTG TAC TGG GAC GTG ATG TTG GAG AA and AGA GTA AAG AGG TGG GAT GGT GAT GG resulting in a 300-bp fragment. The fragment was cloned and sequenced for verification. Hybridization was performed as previously described.<sup>3</sup> BAC 138K24 gave a positive signal which was verified by *ZNF331*-specific PCR, and sequence analysis of the PCR product. A 4 ng/μl volume of dig-labelled BAC 138K24 DNA (Dig-Nick-Translation-Kit; Roche Diagnostics, Mannheim, Germany) was used as probe for fluorescence *in situ* hybridization (FISH) in a hybridization



**Figure 1** Canine metaphase spread after GTG-banding (a) and the same metaphase after FISH with BAC 138K24 showing signal on both chromosomes 1q33 (b).

mixture also containing 1 µg/µl salmon sperm DNA, 20 ng/µl sonicated dog DNA, 1 × SSC, 1 × SSPE, 50% formamide and 10% dextran sulphate. FISH was performed using the protocol of Fischer *et al.*<sup>4</sup> with some modifications<sup>5</sup> on metaphase preparations obtained from blood samples of different dogs. FISH analyses were performed after GTG banding of the same metaphase cells. Counterstaining of the chromosomes was carried out using propidium iodide/antifade solution. G-banded chromosomes were identified according to Reimann *et al.*<sup>6</sup> Sixteen metaphases were examined and all showed a signal on CFA1q33 on both chromatids of both chromosomes 1 (Fig. 1). According to previous mapping data,<sup>7</sup> this region is homologous to HSA19q13. Furthermore, two genes, i.e. *CRX* and *GRLF1*, located on HSA19q13.3, recently have been mapped to the telomeric region of CFA1.<sup>8,9</sup>

*Comments:* During the past few years, the dog has become an interesting model organism for several human diseases and tumours. Cytogenetic hotspots in canine tumours that have been found in the dog genome so far include chromosomes 1, 19 and 25 which are preferentially involved in chromosomal fusions.<sup>10</sup> Aberrations in tumours of the dog involving chromosome 1 were described earlier by several authors in various tumours of the dog such as leukaemias, melanomas and breast cancer.<sup>11–14</sup> With the assignment of *ZNF331* to CFA1q33, a region of frequent breaks in human follicular thyroid adenomas has been mapped in the canine genome.

*Accession numbers:* human *ZNF331*: NM\_018555; canine *ZNF331*: AY375188.

## References

- Rippe V. *et al.* (1999) *Genes Chromosomes Cancer* **26**, 229–36.
- Meiboom M. *et al.* (2003) *Cytogenet Genome Res* **101**, 113–7.
- Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94**, 194–5.
- Fischer P. *et al.* (1996) *Mamm Genome* **7**, 37–41.
- Murua Escobar H. *et al.* (2003) *Cytogenet Genome Res* **101**, 33–8.
- Reimann N. *et al.* (1996) *Cytogenet Cell Genet* **73**, 140–4.
- Yang F. *et al.* (1999) *Genomics* **62**, 189–202.
- Akhmedov N.B. *et al.* (2002) *Mol Vis* **8**, 79–84.
- Zangerl B. *et al.* (2002) *Gene* **294**, 167–76.
- Reimann N. *et al.* (1999) *J Natl Cancer Inst* **91**, 1688–9.
- Bartnitzke S. *et al.* (1992) *Cytogenet Cell Genet* **60**, 135–7.
- Mayr B. *et al.* (1993) *Vet Pathol* **30**, 311–3.
- Reimann N. *et al.* (1998) *Cancer Genet Cytogenet* **101**, 49–52.
- Horsting N. *et al.* (1999) *Res Vet Sci* **67**, 149–51.

*Correspondence:* Dr J. Bullerdiek (bullerd@uni-bremen.de)

doi:10.1111/j.1365-2052.2004.01147.x

## Linkage mapping of ovine cysteine and histidine-rich protein gene (*CYHR1*) to chromosome 9

**J. H. Calvo\***, **S. Marcos\***, **A. E. Beattie<sup>†</sup>**, **J. J. Jurado\*** and **M. Serrano\***

\*Departamento de Mejora Genética Animal, INIA, Madrid, Spain.

<sup>†</sup>AgResearch Molecular Biology Unit, Department of Biochemistry and Centre for Gene Research, University of Otago, Dunedin, New Zealand. Present address: J. H. Calvo, Unidad de Tecnología en Producción Animal, CITA-Gobierno de Aragón, Zaragoza, Spain

Accepted for publication 12 April 2004

*Source/description:* Cysteine and histidine-rich cytoplasmic protein (CYHR1) is involved in cellular trafficking transport of galectin 3.<sup>1</sup> Furthermore, CYHR1 has a broad range of biological activities including DNA and RNA binding, enzyme catalysis, protein–protein interactions, and signal transduction, because it contributes a metal-binding domain multimeric protein. In cattle, the gene encoding this protein has been mapped in the centromeric region of BTA14 (at 8 cM proximal to *CSSM66*) and linkage disequilibrium between the bovine *CYHR1* gene and a QTL with significant effects on milk, fat and protein yield has been demonstrated.<sup>2</sup>

# VIII.

## Expression pattern of the *HMGB1* gene in sarcomas of the dog

Meyer B, Murua Escobar H, Hauke S, Richter A, Winkler S, Rogalla  
P, Flohr AM, Bullerdiek J, Nolte I

*Anticancer Res. 2004; 24(2B): 707-10.*

### Contribution to the work

- Planning and coordination of all preliminary work
- Radioactive probe labelling
- Northern Blot Hybridisation
- Blot imaging

## Expression Pattern of the *HMGB1* Gene in Sarcomas of the Dog

BRITTA MEYER<sup>1</sup>, HUGO MURUA ESCOBAR<sup>2,3</sup>, SVEN HAUKE<sup>1</sup>, ANDREAS RICHTER<sup>1</sup>, SUSANNE WINKLER<sup>3</sup>, PIERE ROGALLA<sup>1</sup>, ALJOSCHA M. FLOHR<sup>3</sup>, JÖRN BULLERDIEK<sup>3</sup> and INGO NOLTE<sup>2</sup>

<sup>1</sup>*alcedo biotech GmbH, Leobener Strasse ZHG, D-28359 Bremen;*

<sup>2</sup>*Clinic for Small Animals, School of Veterinary Medicine, Bischofsholer Damm 15, D-30173 Hannover;*

<sup>3</sup>*Center for Human Genetics, University of Bremen, Leobener Strasse ZHG, D-28359 Bremen, Germany*

**Abstract.** *Background: The human high mobility group protein B1 (HMGB1) has attracted considerable interest among oncologists because it sensitises cancer cells to the anticancer drug cisplatin by shielding cisplatin-DNA adducts from nucleotide excision repair. Materials and Methods: Since cisplatin is the cornerstone of adjuvant systemic therapy for osteosarcomas, in both humans and dogs, the expression pattern of the HMGB1 gene in seven canine sarcomas was investigated by Northern blot analysis and semi-quantitative RT-PCR. Results: A strong intertumoural variation of HMGB1 expression was detected by Northern blot analysis and confirmed by the semi-quantitative RT-PCR established herein. Conclusion: The observed variations of HMGB1 expression in canine sarcomas emphasises the role of HMGB1 as a potential marker of clinical interest as its expression level may predict the clinical outcome of therapies based on cisplatin. The semi-quantitative RT-PCR established allows a quick and convenient determination of the HMGB1 expression level as necessary for clinical applications.*

The related platinum compounds cisplatin and carboplatin are widely used antitumour drugs for the treatment of a number of malignancies. The main cytotoxic effect of cisplatin/carboplatin is the formation of cisplatin/carboplatin-DNA adducts characterised by intrastrand cross-links and significantly bended and distorted DNA.

Gel mobility shift assays revealed a selective affinity of high mobility group (HMG) proteins for cisplatin-DNA adducts (1). The recognition of cisplatin damage by HMG is assumed to mediate cisplatin cytotoxicity. HMG proteins

are chromatin-associated non-histone proteins characterised by low molecular weight, acid-solubility and a high content of charged amino acids. According to their molecular size, sequence and DNA binding capacity, three families have been distinguished: HMGB (formerly HMG1/2), HMGN (formerly HMG14/17) and HMGA (formerly HMGI(Y)) (2,3). The HMGB family, comprising HMGB1, HMGB2 and HMGB3, is characterised by its two DNA-binding domains called the "HMG-Box" (4,5).

One of the best analysed members of the group of HMG-Box proteins is HMGB1 (synonymously known as HMG1 or amphoterin). Both DNA-binding domains selectively bind with a very high affinity to major cisplatin-DNA adducts (6-8) and interaction between HMGB1 and cisplatin-damaged DNA contributes to its biological activity, as it sensitises cancer cells to cisplatin by shielding its major DNA adducts from nucleotide excision repair (9,10).

Interestingly, *HMGB1* gene expression can be induced by oestrogens in breast cancer cells probably due to an up-regulation of the gene, so that *HMGB1* itself can be considered an oestrogen-responsive gene (11). Recently, we were able to explain this observation by the identification of two oestrogen responsive elements within the first intron of *HMGB1* (12). He *et al.* (10) have shown that, in oestrogen receptor-positive human breast cancer cells, oestrogen can significantly increase the effect of cisplatin by causing an overexpression of *HMGB1*. This finding has led to the conclusion that oestrogen treatment prior to cisplatin therapy may sensitise the cancer cells against that drug. Accordingly, a clinical trial for the treatment of gynaecological tumours with cisplatin has already been approved by the Food and Drug Administration (FDA) (10). On the other hand, the former experiment clearly shows that the quantitation of the intratumoural *HMGB1* expression level may be of high impact for a cisplatin/carboplatin therapy for two reasons. Firstly, it may predict the clinical outcome of the therapy; secondly, it may influence the therapy protocol as, for example, tumours

*Correspondence to:* Prof. Dr. Ingo Nolte, Clinic for Small Animals, School of Veterinary Medicine, Bischofsholer Damm 15, D-30173 Hannover, Germany. Tel: +49-511-8567251, Fax: +49-511-8567686, e-mail: inolte@klt.tiho-hannover.de

*Key Words:* Osteosarcoma, cisplatin, *HMGB1* expression, semi-quantitative RT-PCR.

Table I. Sarcoma samples analysed in this study.

Sarcoma sample	Tumour	Breed	Sex	Age
OS1	Osteosarcoma	Rottweiler	f	1 yr
OS2	Osteosarcoma	Crossbreed	f	4 yrs
OS3	Osteosarcoma	German Shepherd	m	6 yrs
OS4	Osteosarcoma	Crossbreed	m	9 yrs
OS5	Osteosarcoma	German Shepherd	m	n.r. <sup>1</sup>
FS	Fibrosarcoma	Bobtail	m	5 yrs
LMS	Leiomyosarcoma	Crossbreed	f	10 yrs

<sup>1</sup>n.r. = not reported

showing a high *HMGB1* expression level may be treated with a lower amount of this antitumour drug.

Due to the close similarities of numerous canine diseases to their human counterparts, the role of the dog as a model organism for therapeutic approaches is justified. Furthermore, genes and proteins known to be of high diagnostic and therapeutic impact in man can also be considered to play an important role in the dog.

Osteosarcomas and several types of carcinomas belong to the group of canine malignancies often treated with cisplatin or carboplatin. So far no data are available analysing the expression pattern of the *HMGB1* gene in canine sarcomas. Thus, in this study we analysed the *HMGB1* expression level in five canine osteosarcomas, one fibrosarcoma and one leiomyosarcoma by Northern blot experiments. Based on the observed strong intertumoural variation of *HMGB1* expression, we further established a quick RT-PCR-based diagnostic system for future studies.

## Materials and Methods

**Tissue samples.** All canine tumour samples used in this study (Table I) were provided by the Clinic for Small Animals, Hanover, Germany. Samples were taken during surgery, immediately frozen in liquid nitrogen and stored at -80°C.

**RNA isolation.** Total RNA extraction of the canine sarcoma samples was performed according to the RNeasy midi protocol for isolation of total RNA from heart, muscle and skin tissue (Qiagen, Hilden, Germany) including a Proteinase K digest. Enrichment of poly A<sup>+</sup> mRNA was carried out using the Oligotex mRNA kit (Qiagen).

**Northern blot hybridisation.** For Northern blot analysis, 5 µg of mRNA from each sample were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto a Hybond-XL charged nylon membrane (Amersham Biosciences, Buckinghamshire, England) by capillary blot overnight. As a probe for hybridisation, a 603 bp cDNA fragment derived from the ORF (exon 2-5) of the canine *HMGB1* gene was generated by PCR using the primer pair ToastUP (5' GGGCAAAGGAGATCCTAAGAAG 3') (13) and Ex5lo (5'

TCTTCCTCCTCCTCATCC 3'). A 445 bp cDNA probe detecting the 1.3 kb transcript of the canine *GAPDH* gene was amplified by PCR with the primer set GAPDH2up (5' GTGAAGGTCGGAGTCAAC 3') and GAPDHdog5do (5' AGGAGGCATTGCTGACAAT 3'). Probes were labelled with 50 µCi(α-<sup>32</sup>P)dCTP (Amersham Biosciences) using the Megaprime Labelling Kit (Amersham Biosciences) for random-primed labelling (14). Hybridisation was performed for 3 h at 68°C in 10 ml of PerfectHyb Plus Hybridisation Buffer (Sigma-Aldrich, Saint Louis, USA). The membranes were washed for 5 min with low stringency at RT in 2x SSC, 0.1% SDS and twice for 20 min with high stringency at 68°C in 0.5x SSC, 0.1% SDS. Signals were visualised using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA). Quantitation of the transcripts of *HMGB1* and *GAPDH* was performed using the software program ImageQuant (Molecular Dynamics).

**Semi-quantitative RT-PCR.** cDNA synthesis was performed using primer AP2 (5' AAGGATCCGTCGACATCT<sub>(17)</sub> 3') with 500 ng of mRNA with SuperScript Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. In order to determine the expression of *HMGB1* in relation to that of the housekeeping gene *GAPDH*, a duplex PCR was established using the primer sets ToastUP/Ex5lo and GAPDH2up/GAPDHdog5do (see above). PCR reactions were set up according to the "basic PCR protocol" of Taq DNA Polymerase (Invitrogen) using the following PCR program: initial denaturation for 5 min at 94°C, 28 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 55°C and extension for 45 sec at 72°C, followed by a final extension for 10 min at 72°C. The appropriate number of cycles was previously determined so that for both PCR-products amplification was in the exponential range (data not shown). PCR-products were separated on a 1.2% agarose gel stained with VistraGreen (Amersham) and visualised using a Storm PhosphorImager (Molecular Dynamics). Quantitation of the PCR-fragments of *HMGB1* and *GAPDH* was performed using the software program ImageQuant (Molecular Dynamics) measuring pixel intensities.

## Results

Northern blot hybridisation on a series of 5 osteosarcomas, one fibrosarcoma and one leiomyosarcoma sample of the dog (Table I), using a cDNA probe derived from the ORF (Exon 2-5) of the canine *HMGB1* gene, resulted in the detection of two *HMGB1* mRNA transcripts of approximately 1.4 and 2.4 kb (Figure 1), which are similar to that observed in human tissues (15-17) and various canine tissues (18). In order to quantify the expression of *HMGB1*, the blot was rehybridised with a canine *GAPDH*-specific cDNA probe (Figure 1). Summing up the intensities of the 1.4 and 2.4 kb *HMGB1* signals, the *HMGB1*-RNA / *GAPDH*-RNA ratios were calculated. As shown in Figure 1, the analysed canine sarcoma samples revealed a strong intertumoural variation in the relative expression of *HMGB1*. Values obtained by Northern blot analysis for the osteosarcoma samples varied between 0.52 and 1.31, while the fibrosarcoma and the leiomyosarcoma showed ratios of 0.73 and 0.24, respectively (Table II).

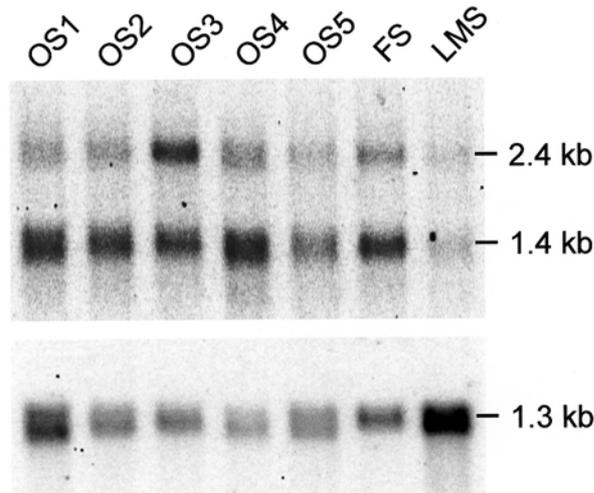


Figure 1. Northern blot analysis of five osteosarcomas (OS1-5), one fibrosarcoma (FS) and one leiomyosarcoma (LMS) of the dog hybridised with a *HMGB1*-specific cDNA probe detecting the two canine *HMGB1* transcripts of approximately 1.4 and 2.4 kb (upper part). Co-hybridisation of the same membrane with a *GAPDH*-specific cDNA probe detecting a 1.3 kb transcript (lower part).

Table II. Absolute and relative *HMGB1*-mRNA / *GAPDH*-mRNA ratios.

Sarcoma sample	Absolute <i>HMGB1</i> / <i>GAPDH</i> -RNA ratios		Relative <i>HMGB1</i> / <i>GAPDH</i> -RNA ratios <sup>1</sup>	
	RT-PCR	Northern blot	RT-PCR	Northern blot
OS1	0.95	0.52	1.09	0.7
OS2	0.99	0.79	1.13	1.06
OS3	1.02	1.05	1.17	1.41
OS4	1.28	1.31	1.47	1.75
OS5	0.72	0.60	0.83	0.79
FS	0.73	0.73	0.84	0.97
LMS	0.42	0.24	0.48	0.32
Mean value	0.87	0.75	1.0	1.0

<sup>1</sup> Calculated with the mean values of the absolute *HMGB1* / *GAPDH*-RNA ratios set to one.

In order to confirm the results and to develop a less time- and material-consuming technique, we established a semi-quantitative duplex RT-PCR suitable for detecting intertumoural variation of *HMGB1* expression in relation to expression of the house-keeping gene *GAPDH* (Figure 2). After quantitation of the signals obtained by RT-PCR, the *HMGB1*-RNA / *GAPDH*-RNA ratios were calculated. The values for the osteosarcoma samples varied between 0.72 and 1.28, while the ratios for the fibrosarcoma and the leiomyosarcoma were 0.73 and 0.42, respectively (Table II). In order to determine the comparability of the results

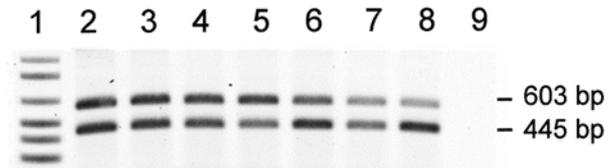


Figure 2. Semi-quantitative duplex RT-PCR products of *HMGB1* (603 bp) and *GAPDH* (445 bp) using canine cDNAs of five osteosarcomas, one fibrosarcoma and one leiomyosarcoma after electrophoresis and *VistraGreen* staining (Amersham Biosciences). Lane 1: DNA molecular weight standard 1 Kb Plus DNA Ladder (Invitrogen). Lanes 2-6: osteosarcoma samples 1-5 (OS1-5). Lane 7: fibrosarcoma sample (FS). Lane 8: leiomyosarcoma sample (LMS). Lane 9: H<sub>2</sub>O, negative control.

obtained by the Northern blot hybridisation and RT-PCR analyses, mean values for each test series were calculated, set to one, and relative expression levels were determined (Table II, Figure 3). Statistical analysis using the Pearson's Correlation Test revealed a significant correlation between the relative *HMGB1* expression level obtained by Northern blot hybridisation and the level obtained by the established RT-PCR ( $r=0.8919$ ,  $p=0.0071$ ).

## Discussion

Cisplatin and carboplatin are widely used anticancer drugs, manifesting their cytotoxicity to tumour cells by damaging DNA, generating a distorted DNA duplex. *HMGB1* proteins selectively bind with high affinity to cisplatin or carboplatin-DNA adducts and several investigations revealed that this interaction contributes to tumour death by blocking excision repair of the major cisplatin-DNA adducts (9,10).

No features have been identified yet allowing clinicians to predict the response to cisplatin or carboplatin therapies in dogs with osteosarcomas at the time of diagnosis or during treatment (19). Hence, it was the aim of this study to analyse the expression level of *HMGB1* in canine sarcomas.

Based on Northern blot and RT-PCR analyses, we were able to show an intertumoural variation of *HMGB1* expression levels among canine sarcomas. Very recently, comparable results were obtained for human breast cancer samples (17,20) and a clinical trial designed to increase *HMGB1* expression by oestrogen treatment has been approved by the FDA (10). The observed intertumoural variances of *HMGB1* expression in seven sarcomas analysed in this study may be of importance for therapeutic approaches based on cisplatin/carboplatin treatment as, for example, tumours showing a high *HMGB1* expression level may be treated with a lower amount of this antitumour drug. However, future clinical studies including a greater number of tumours have to be performed to correlate the

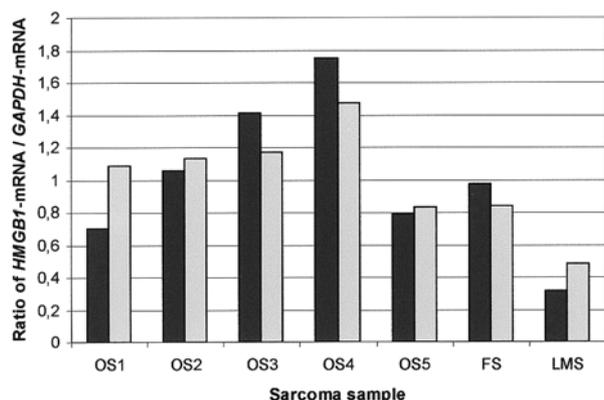


Figure 3. Variation of relative *HMGB1* expression in five osteosarcomas (OS1-5), one fibrosarcoma (FS) and one leiomyosarcoma (LMS) of the dog as revealed by Northern blot analysis (dark grey bars) and semi-quantitative RT-PCR (light grey bars). In order to compare the results obtained by the two methods, mean values for each test series were calculated, set to one and relative expression levels were determined.

*HMGB1* expression level with clinical outcome of cisplatin/carboplatin chemotherapy. The statistically significant correlation of the relative *HMGB1* expression levels obtained by Northern blot analyses as well as duplex RT-PCR makes the established PCR approach a quick and convenient method to determine the intratumoural *HMGB1* expression.

## References

- Kartalou M and Essigmann JM: Recognition of cisplatin adducts by cellular proteins. *Mutat Res* 478: 1-21, 2001.
- Bustin M, Lehn DA and Landsman D: Structural features of the HMG chromosomal proteins and their genes. *Biochim Biophys Acta* 1049: 231-243, 1990.
- Bustin M and Reeves R: High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. *Prog Nucleic Acid Res Mol Biol* 54: 35-100, 1996.
- Jantzen HM, Admon A, Bell SP and Tjian R: Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature* 344: 830-836, 1990.
- Landsman D and Bustin M: A signature for the HMG-1 box DNA-binding proteins. *Bioessays* 15: 539-546, 1993.
- Pil PM and Lippard SJ: Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science* 256: 234-237, 1992.
- Jung Y and Lippard SJ: Nature of full-length HMGB1 binding to cisplatin-modified DNA. *Biochemistry* 42: 2664-2671, 2003.
- Kasparkova J, Delalande O, Stros M, Elizondo-Riojas M-A, Vojtiskova M, Kozelka J and Brabec V: Recognition of DNA interstrand cross-link of antitumor cisplatin by HMGB1 protein. *Biochemistry* 42: 1234-1244, 2003.
- Zamble DB, Mu D, Reardon JT, Sancar A and Lippard SJ: Repair of cisplatin-DNA adducts by the mammalian excision nuclease. *Biochemistry* 35: 10004-10013, 1996.
- He Q, Liang CH and Lippard SJ: Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. *Proc Natl Acad Sci USA* 97: 5768-5772, 2000.
- Chau KY, Lam HY and Lee KL: Estrogen treatment induces elevated expression of HMG1 in MCF-7 cells. *Exp Cell Res* 241: 269-272, 1998.
- Borrmann L, Kim I, Schultheiss D, Rogalla P and Bullerdiek J: Regulation of the expression of HMG1, a co-activator of the oestrogen receptor. *Anticancer Res* 21: 301-305, 2001.
- Jiang Z, Priat C and Galibert F: Traced orthologous amplified sequence tags (TOASTs) and mammalian comparative maps. *Mamm Genome* 9: 577-787, 1998.
- Feinberg AP and Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6-13, 1983.
- Wen L, Huang JK, Johnson BH and Reeck GR: A human placental cDNA clone that encodes nonhistone chromosomal protein HMG-1. *Nucleic Acid Res* 17: 1197-1214, 1989.
- Rogalla P, Kazmierczak B, Flohr AM, Hauke S and Bullerdiek J: Back to the roots of a new exon—the molecular archaeology of a SP100 splice variant. *Genomics* 63: 117-122, 2000.
- Flohr AM, Rogalla P, Meiboom M, Borrmann L, Krohn M, Thode-Halle B and Bullerdiek J: Variation of HMGB1 expression in breast cancer. *Anticancer Res* 21: 3881-3885, 2001.
- Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM, Bullerdiek J and Nolte I: Molecular characterization of the canine HMGB1. *Cytogenet Genome Res* 101: 33-38, 2003.
- Hahn KA, Legendre AM and Talbott JR: The frequency of micronuclei in lymphocytes of dogs with osteosarcomas: a predictive variable for tumor response during cisplatin chemotherapy. *Cancer Epidemiol Biomarkers Prev* 5: 653-656, 1996.
- Brezniceanu M-L, Völp K, Bösse S, Solbach C, Lichter P, Joos S and Zörnig M: HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast cancer carcinoma. *FASEB J* 17: 1295-1297, 2003.

Received July 9, 2003

Revised November 4, 2003

Accepted December 12, 2003

# **IX.**

## **The HOPE-technique permits Northern blot and microarray analyses in paraffin-embedded tissues**

**Goldmann T, Flohr AM, Murua Escobar H, Gerstmayer B, Janssen  
U, Bosio A, Loeschke S, Vollmer E, Bullerdiek J**

*Pathol Res Prac. 2004; 200: 511-5.*

Contribution to the work

- Radioactive probe labelling
- Northern Blot Hybridisation
- Blot imaging



## ORIGINAL ARTICLE

**The HOPE-technique permits Northern blot and microarray analyses in paraffin-embedded tissues**

Torsten Goldmann<sup>a,1</sup>, Aljoscha M. Flohr<sup>b,1</sup>, Hugo Murua Escobar<sup>b,c</sup>,  
Bernhard Gerstmayer<sup>d</sup>, Uwe Janssen<sup>d</sup>, Andreas Bosio<sup>d</sup>, Siegfried Loeschke<sup>b</sup>,  
Ekkehard Vollmer<sup>a</sup>, Jörn Bullerdiek<sup>b,\*</sup>

<sup>a</sup> *Clinical and Experimental Pathology, Research Center Borstel, Parkallee 3, 23845 Borstel, Germany*

<sup>b</sup> *Center for Human Genetics and Genetic Counseling, Molecular Oncol and Tumor Cyrogenetics, University of Bremen, Leobenerstr Straße ZHG, D-28359 Bremen, Germany*

<sup>c</sup> *School of Veterinary Medicine Hanover, Clinic for Small Animals, Bischofsholer Damm 15, 30173 Hannover, Germany*

<sup>d</sup> *Memorec Stoffel GmbH, Stöckheimer Weg 1, 50829 Köln, Germany*

Received 8 January 2004; accepted 23 March 2004

**Abstract**

There is an increasing demand for tissue samples that, after having been used for conventional histologic examination, are also suited for molecular analyses. As to formalin-fixed, paraffin embedded (FFPE) tissue, the latter applications are very limited. The HOPE (*Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect*) technique comprises a new protection-solution with an organic buffer, with acetone as the only dehydrating agent, and pure paraffin of 52–54°C melting temperature, allowing for all pathologic routine investigations. In contrast to FFPE tissue, the HOPE-technique allows for the application of molecular methods, such as high molecular DNA and RNA isolation, which can be used for PCR and reverse transcription PCR (RT-PCR). In this study, we investigated whether RNA from HOPE-fixed tissue samples is suitable for Northern blot and microarray analyses. RNAs of two HOPE-fixed breast cancer specimens of different histologic grade were used to carry out an array experiment. It turned out that RNA from HOPE-fixed tissue is of high quality and can be successfully used for array experiments. In addition, by detecting *GAPDH* and high mobility group protein gene B1 (*HMGB1*)-specific transcripts, we were able to demonstrate that RNA from HOPE-fixed tissue can also be used for Northern blot hybridization.

© 2004 Elsevier GmbH. All rights reserved.

**Keywords:** HOPE; RNA; Northern blot; Microarray; Breast cancer

**Introduction**

Formalin-fixed, paraffin embedded (FFPE) tissue is the material most commonly used for pathologic and

molecular pathologic applications. However, FFPE tissue is often not suited for downstream applications, such as high molecular DNA and RNA isolation. Accordingly, an alternative fixative that allows for the preservation of RNA in embedded tissue samples is of great value for molecular pathologic investigations, such as reverse transcription PCR (RT-PCR), RNA in situ hybridization (ISH), Northern blot and microarray analyses. The HOPE (*Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect*) technique

\*Corresponding author. Tel.: +49-421-218-3589; fax: +49-421-218-4239.

E-mail address: [bullerdiek@uni-bremen.de](mailto:bullerdiek@uni-bremen.de) (J. Bullerdiek).

<sup>1</sup>Torsten Goldmann and Aljoscha M. Flohr have contributed equally to this article.

comprises a new protection-solution with an organic buffer, with acetone as the only dehydrating agent, and pure paraffin of 52–54°C melting temperature allowing for pathologic routine investigations, i.e., tissue fixation, subsequent paraffin-embedding, and sectioning [10]. It has been shown that it is generally possible to extract high molecular weight DNA and RNA suitable for downstream applications, such as PCR, RT-PCR, and ISH detecting mRNA from HOPE-fixed, paraffin embedded tissue samples [3,7,14]. It is also possible to carry out immunohistochemistry using HOPE-fixed tissue samples [3]. Umland et al. [12] found that HOPE fixation can be applied to ISH and immunocytochemistry (ICC) on cultured cells. Furthermore, it was recently demonstrated that the use of the HOPE-technique improved the detectability of biomarkers relevant for diagnoses [8]. The aim of this study was to demonstrate that RNA isolated from HOPE-fixed tissue can also be used for Northern blot and microarray hybridization.

## Materials and methods

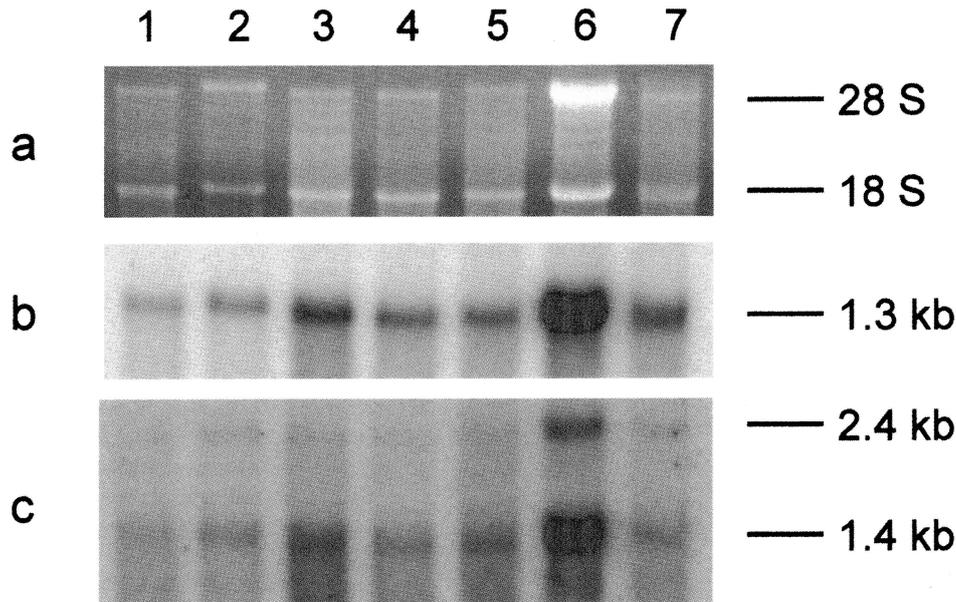
Seven human breast cancer samples were HOPE-fixed and paraffin-embedded as described previously [10]. After deparaffination of 20–30 5 µm sections per specimen, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RNA was quantified by a photometer, and integrity was checked by agarose gel electrophoresis revealing the 28S and 18S rRNA bands. For Northern blot hybridization, 20 µg total RNA of each sample was separated on a 1.2% (w/v) denaturing agarose gel containing 0.67% (v/v) formaldehyde. RNAs were transferred onto Hybond-XL positively charged nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) by capillary blot. A 445 bp cDNA fragment of the *GAPDH* gene and a 659 bp fragment covering the complete open reading frame of the high mobility group protein gene B1 (*HMGB1*) gene served as molecular probes for hybridization. The *GAPDH*-specific probe was generated by RT-PCR using HeLa cDNA and the primer pair GAPDH2.up (5'-GTG AAG GTC GGA GTC AAC G-3') and GAPDH5.do (5'-AGG AGG CAT TGC TGA TGA T-3'). The *HMGB1*-specific probe was generated by RT-PCR using the cloned PCR product as template and the primer pair ORF.up (5'-AAT AAC TAA ACA TGG GCA AAG GA-3') and ORF.do (5'-TTA TTC ATC ATC ATC ATC TTC TTC TT -3'). Probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Megaprime DNA labeling system (Amersham Biosciences, Buckinghamshire, England) according to the random primer

extension protocol [5]. Using the PerfectHyb Plus hybridization solution (Sigma, Saint Louis, USA), prehybridization was carried out for 5 min, and hybridization for 2.5 h at 68°C. The membranes were washed twice for 5 min at room temperature with a low stringency wash buffer (2 × SSC, 0.1% SDS), and twice for 20 min at 68°C with a high stringency wash buffer (0.5 × SSC, 0.1% SDS). Signals were visualized by a STORM imager (Molecular Dynamics, Sunnyvale, USA).

We used RNAs isolated from two HOPE-fixed invasive ductal breast carcinomas: a grade 2 tumor (age of patient: 61 years) and a grade 3 tumor (age of patient: 50 years) for array hybridization. For the microarray hybridization, the qualitative integrity test of the two purified total RNA samples and linearly amplified RNA (aRNA), as well as quantitative measurements, were done with capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and a spectrometer. Two µg of total RNA obtained from two different breast cancer samples (grades 2 and 3) was subjected to a modified linear amplification protocol [4]. Of each aRNA, 2 µg was labeled by RT with Cy5 (red) and Cy3 (green) fluorescence. Fluorescently labeled samples were purified, combined, and subsequently subjected to a predefined PIQOR™ Immuno/Oncology microarray consisting of 642 oncology-relevant human cDNAs (for a complete list of genes see [www.memorec.com](http://www.memorec.com), memorec stoffel gmbh, Cologne, Germany). Bioinformatically selected cDNA fragments containing no repetitive elements, with minimal homology to any other gene in the data bases (<85% homology) and having an average length of 200–400 bp [11], were generated by RT-PCR (Superscript™II, Invitrogen, Groningen, The Netherlands) using gene-specific primers, cloned into pGEM®-T vector (Promega, Mannheim, Germany), and sequence-verified. Inserts were amplified using vector-sequence-derived primers with the sense primer carrying a 5'-amino-modification. Amplified inserts were purified (Qiaquick 96 PCR BioRobot Kit, Qiagen, Hilden, Germany) and checked on an agarose gel. Finally, cDNAs were diluted in a buffer containing 30 × SSC, 1 M betain to reach a final concentration of 3 × SSC and 0.1 M betain and a cDNA concentration of 100 ng/µl. Each cDNA was spotted as quadruplicates on coated glass slides. Hybridization, scanning, and data analysis were performed as described in detail previously [1].

## Results

Qualitative integrity test by capillary electrophoresis and spectrometer revealed that the two RNA samples



**Fig. 1.** (a) Denaturing agarose gel electrophoresis of seven RNAs isolated from HOPE-fixed breast cancer specimens revealing the 18S and 28S rRNA bands. (Lanes 1–5, and 7: invasive ductal carcinoma, lane 6: medullary carcinoma.) (b) Northern blot analysis detecting a 1.3 kb transcript of the *GAPDH* gene using 20  $\mu$ g of total RNA isolated from seven HOPE-fixed, paraffin-embedded breast cancer specimens. (c) Northern blot analysis detecting the two splice variants of 1.4 and 2.4 kb of the *HMGB1* gene in the same breast cancer specimens.

used for array hybridization showed two prominent peaks within the elution profile, corresponding to 28S and 18S rRNA. The ratio of 28S to 18S rRNA peak areas was  $>1.5$ , confirming the integrity of the RNA (ratios  $<1$  indicate that RNA is being degraded). Likewise, linearly amplified RNA showed a broad distribution spectrum of RNAs with different lengths as expected. The sizes of the amplified RNAs ranged from 0.2 to 3.0 kb, with an average length of 1.0 kb, indicating a good representation of the different mRNA species initially isolated from the biopsies.

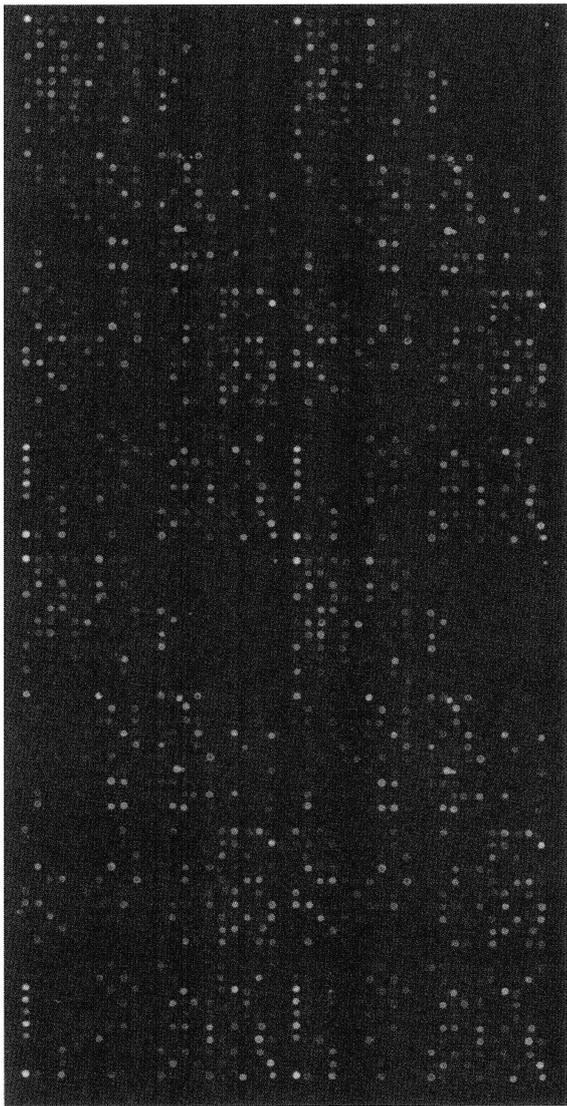
Cy3 and Cy5 fluorescently labeled cDNAs were combined and simultaneously hybridized to a predefined microarray, comprising 642 oncologically relevant genes, to determine whether or not this RNA is suitable for array experiments. It turned out that RNA prepared from HOPE-fixed tissue can be successfully used for microarray hybridization (Fig. 2). We found that 46 of 642 genes were  $>$ two-fold upregulated, and that 45 of 642 genes were  $>$ two-fold downregulated when hybridization results of grade 3 versus grade 2 samples were compared with each other.

In addition, we were able to perform Northern blot analyses using total RNA isolated from seven HOPE-fixed breast cancer samples. Integrity of RNA samples was checked by standard gel electrophoresis revealing the 18S and 28S rRNA bands (Fig. 1a). In all breast

cancer RNAs, a *GAPDH*-specific transcript of 1.3 kb (Fig. 1b) and the two previously described splice variants of 1.4 and 2.4 kb of the *HMGB1* could be detected by Northern blot hybridization (Fig. 1c) [6,13]. These results match a previous study detecting *HMGB1* expression in nitrogen-frozen primary breast cancer samples [6]. By contrast, RNA extracted from FFPE tissue is significantly degraded, and amplification of cDNA fragments longer than 200 bp is usually unsuccessful [9].

## Discussion

In this study, we found that RNA isolated from HOPE-fixed, paraffin embedded tissue is generally suitable for Northern blot and microarray hybridization. To the best of our knowledge, this is the first study to describe this novel methodology. However, owing to the small number of specimens tested, the convincing initial results presented here need to be verified by large-scale studies. Using the HOPE-technique for tissue fixation and preservation, the pathological archives would become a unique source of defined biopsies derived from normal and pathologically altered tissues for which extensive clinical data are available. It would be possible to conduct



**Fig. 2.** False color overlay of a hybridized PIQOR™ Immuno/Onco array after scanning the spotted area at 543 and 633 nm. aRNA from the grade 2 carcinoma was labeled with Cy3 (green), and the grade 3 carcinoma was labeled with Cy5 (red).

molecular pathologic investigations based on microarray analyses that aim at detecting differential gene expression patterns and reliable gene expression studies by Northern blot in pathologically altered tissue samples. Gene expression microarray technology is increasingly finding use for determining the expression of virtually all human genes at the mRNA level, and the use of microarrays is predicted to become an essential routine tool for cancer and biomedical research [2]; however, its application to FFPE tissue samples does not result in sufficiently reproducible results. Thus, the HOPE-technique constitutes an interesting procedure allowing for retrospective molecular analyses as well.

## Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (German Research Foundation) (Bu 592/4-3). We thank Mrs. Heike Kuehl for her excellent technical assistance.

## References

- [1] A. Bosio, C. Knorr, U. Janssen, S. Gebel, H.J. Haussmann, T. Muller, Kinetics of gene expression profiling in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke, *Carcinogenesis* 23 (2002) 741–748.
- [2] P.A. Clarke, R. te Poele, R. Wooster, P. Workman, Gene expression microarray analysis in cancer biology, pharmacology, and drug development: progress and potential, *Biochem. Pharmacol.* 62 (2001) 1311–1336.
- [3] D. Droemann, T. Goldmann, D. Branscheid, R. Clark, K. Dalhoff, P. Zabel, E. Vollmer, Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung, *Histochem. Cell Biol.* 119 (2003) 103–108.
- [4] J. Eberwine, Amplification of mRNA populations using aRNA generated from immobilized oligo(dT)-T7 primed cDNA, *Biotechniques* 20 (1996) 584–591.
- [5] A.P. Feinberg, B. Vogelstein, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem.* 132 (1983) 6–13.
- [6] A.M. Flohr, P. Rogalla, M. Meiboom, L. Borrmann, M. Krohn, B. Thode-Halle, J. Bullerdiek, Variation of *HMGB1* expression in breast cancer, *Anticancer Res.* 21 (2001) 3881–3885.
- [7] T. Goldmann, K.H. Wiedorn, H. Kuhl, J. Olert, D. Branscheid, D. Pechkovsky, G. Zissel, J. Galle, J. Muller-Quernheim, E. Vollmer, Assessment of transcriptional gene activity in situ by application of HOPE-fixed, paraffin-embedded tissues, *Pathol. Res. Pract.* 198 (2002) 91–95.
- [8] T. Goldmann, E. Vollmer, J. Gerdes, What's cooking? Detection of important biomarkers in HOPE-fixed paraffin embedded tissues eliminates the need for antigen retrieval, *Am. J. Pathol.* 163 (2003) 2638–2640.
- [9] F. Lewis, N.J. Maughan, V. Smith, K. Hillan, P. Quirke, Unlocking the archive—gene expression in paraffin-embedded tissue, *J. Pathol.* 195 (2001) 66–71.
- [10] J. Olert, K.H. Wiedorn, T. Goldmann, H. Kuhl, Y. Mehraein, H. Scherthan, F. Niketeghad, E. Vollmer, A.M. Müller, J. Müller-Navia, HOPE-fixation: a novel fixing method and paraffin embedding technique for human soft tissues, *Pathol. Res. Pract.* 197 (2001) 823–826.
- [11] S. Tomiuk, K. Hofmann, Microarray probe selection strategies, *Brief Bioinform.* 2 (2001) 329–340.
- [12] O. Umland, A.J. Ulmer, E. Vollmer, T. Goldmann, HOPE fixation of cytospin preparations of human cells for in situ hybridization and immunocytochemistry, *J. Histochem. Cytochem.* 51 (2003) 977–980.
- [13] L. Wen, J.K. Huang, B.H. Johnson, G.R. Reeck, A human placental cDNA clone that encodes nonhistone

- chromosomal protein HMG-1, Nucl. Acids Res. 17 (1989) 1197–1214.
- [14] K.H. Wiedorn, J. Oler, R.A. Stacy, T. Goldmann, H. Kuhl, J. Matthus, E. Vollmer, A. Bosse, HOPE—a new fixing technique enables preservation and extraction of high molecular weight DNA and RNA of > 20 kb from paraffin-embedded tissues. Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect, Pathol. Res. Pract. 198 (2002) 735–740.

# X.

## The canine *HMGA1*

**Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, Bullerdiek J.**

*Gene. 2004; 14(330): 93-9.*

### Contribution to the work

- Planning and coordination of all done work
- Radioactive probe labelling
- Northern Blot Hybridisation
- *In silico* Analysis
- composing of the publication



## The canine *HMGAI*

Hugo Murua Escobar<sup>a,b</sup>, Jan T. Soller<sup>a</sup>, Andreas Richter<sup>a</sup>, Britta Meyer<sup>a</sup>, Susanne Winkler<sup>a</sup>,  
Aljoscha M. Flohr<sup>a</sup>, Ingo Nolte<sup>b</sup>, Jörn Bullerdiek<sup>a,\*</sup>

<sup>a</sup>Centre for Human Genetics, University of Bremen, Leobener Strasse ZHG, D-28359 Bremen, Germany

<sup>b</sup>Small Animal Clinic, School of Veterinary Medicine, Bischofsholer Damm 15, D-30173 Hanover, Germany

Received 28 October 2003; received in revised form 19 December 2003; accepted 15 January 2004

Received by D.A. Tagle

### Abstract

Due to the emerging advantages of numerous canine diseases as a genetic model for their human orthologs, the dog could join the mouse as the species of choice to unravel genetic mechanisms, e.g. of cancer predisposition, development and progression. However, precondition for such studies is the characterisation of the corresponding canine genes.

Human and murine *HMGAI* non-histone proteins participate in a wide variety of cellular processes including regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the induction of neoplastic transformation and promotion of metastatic progression of cancer cells.

Chromosomal aberrations affecting the human *HMGAI* gene at 6p21 were described in several tumours like pulmonary chondroid hamartomas, uterine leiomyomas, follicular thyroid adenomas and others. Over-expression of the proteins of *HMGAI* is characteristic for various malignant tumours suggesting a relation between high titer of the protein and the neoplastic phenotype.

In this study, we characterised the molecular structure of the canine *HMGAI* cDNA, its splice variants and predicted proteins *HMGAIa* and *HMGAIb*. Furthermore, we compared the coding sequence(s) (CDS) of both splice variants for 12 different breeds, screened them for single nucleotide polymorphisms (SNPs) and characterised a basic expression pattern.

© 2004 Elsevier B.V. All rights reserved.

**Keywords:** High mobility group proteins; *HMGAI*; *HMGAIa*; *HMGAIb*; Comparative genomics

### 1. Introduction

As witnessed by a number of recent articles (Kuska, 1996; Kingman, 2000; Ostrander et al., 2000; Vail and

MacEwen, 2000), a growing number of scientists predict that human genetics will focus on the dog in this century (Kuska, 1996). Due to the emerging advantages of numerous canine diseases as a genetic model for their human counterparts, the dog could join the mouse as the species of choice to unravel genetic mechanisms, e.g. of cancer predisposition, development and progression.

The proteins of the human *HMGAI* gene *HMGAIa* and *HMGAIb* are associated with various human diseases including cancer. Due to the similarities of various human and canine cancer entities, the characterisation of the canine *HMGAI* gene could open new fields for experimental and therapeutic approaches.

Four human members of the *HMGAI* protein family are presently known: the *HMGAIa*, *HMGAIb*, *HMGAIc* and *HMGAI2* proteins, which can modify chromatin structure by bending DNA, thus influencing the transcription of a number of target genes. The human *HMGAI* gene on 6p21 encodes the well characterised

**Abbreviations:** A, adenosine; aa, amino acid(s); BAC, bacterial artificial chromosome; bp, base pair(s); cDNA, DNA complementary to RNA; CDS, coding sequence(s); CFA, *Canis familiaris*; Ci, Curie; D, Dalton; dCTP, deoxycytidine 5'-triphosphate; DNA, deoxy-ribonucleic acid; DNase, deoxyribonuclease; G, guanosine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG, high mobility group; *HMGAI*, high mobility group protein A1; *HMGAI2*, high mobility group protein A2; HSA, *Homo sapiens*; M-MLV, Moloney murine leukemia virus; mRNA, messenger ribonucleic acid; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism; UTR, untranslated region.

\* Corresponding author. Tel.: +49-421-2184239; fax: +49-421-2184239.

E-mail address: [bullerdiek@uni-bremen.de](mailto:bullerdiek@uni-bremen.de) (J. Bullerdiek).

HMGA1a and HMGA1b proteins (formerly known as HMGI and HMGY) derived by alternative splicing and the barely characterised HMGA1c variant, while the HMGA2 protein is encoded by a separate gene on chromosome 12 (12q14–15) (for review, Reeves and Beckerbauer, 2001).

Expression of HMGA1 is detectable only at very low levels or is even absent in adult tissues, whereas it is abundantly expressed in embryonic cells (Chiappetta et al., 1996). In humans, 6p21 is often affected by aberrations leading to an up-regulation of *HMGA1* in benign mesenchymal tumours, e.g. lipomas, uterine leiomyomas, pulmonary chondroid hamartomas and endometrial polyps (Williams et al., 1997; Kazmierczak et al., 1998; Tallini et al., 2000). Transcriptional activation due to a chromosomal alteration of *HMGA1* is probably an early and often even primary event of cancer development. In contrast, *HMGA1* expression in malignant epithelial tumours seems to be a rather late event associated with an aggressive behaviour of the tumours. Thus, an over-expression of *HMGA1* was reported for a number of malignancies including thyroid, prostatic, pancreatic, cervical and colorectal cancer (Tamimi et al., 1993; Chiappetta et al., 1995, 1998; Fedele et al., 1996; Bandiera et al., 1998; Abe et al., 1999, 2000). The correlation between *HMGA* expression and tumour aggressiveness in these malignancies has led to the conclusion that *HMGA* expression may present a powerful prognostic molecular marker. The causal role of *HMGA1* expression in the progression of carcinomas has been elucidated by a set of *in vitro* experiments involving *HMGA1* sense and antisense transfection assays (Reeves et al., 2001). An experimental approach aimed at the down-regulation of HMGA protein in tumours has been presented by Scala et al. (2000) who were able to show that an *HMGA1* antisense strategy using an adenoviral vector treatment of tumours induced in athymic mice caused a drastic reduction in tumour size.

Recently, the canine *HMGA1* gene has been mapped to CFA 23. This cytogenetic assignment indicates that the canine *HMGA1* gene does not map to a hotspot of chromosomal breakpoints seen in canine tumours (Becker et al., 2003). However, despite the emerging role of *HMGA1* gene expression in malignancies, the molecular characterisation of the canine *HMGA1* gene had not been carried out before. The characterisation of the molecular structure could permit new therapeutic approaches using the dog as model organism.

In this study, we characterised the molecular structure of the canine *HMGA1* gene on cDNA level, its splice variants and proteins HMGA1a and HMGA1b, and a basic expression pattern. Furthermore, for 12 different canine breeds the coding sequence(s) (CDS) of both splice variants were characterised and screened for SNPs to find out if changes at protein level exist between the different breeds.

## 2. Materials and methods

### 2.1. Tissues

The tissues used in this study were provided by the Small Animal Clinic, Veterinary School, Hanover, Germany. The breeds represented were Alsatian, Bull Terrier, Collie, Dachshund, Doberman Pinscher, German Shorthaired Pointer, Golden Retriever, Jack Russell Terrier, Kangal, Munsterland, West Highland Terrier and Yorkshire Terrier. From each breed up to three samples of testis tissue were taken and used for analyses.

### 2.2. cDNA characterisation

Total RNA was isolated from 150 mg canine testis tissue using TRIZOL LS (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. To avoid genomic DNA contamination a DNase digest of each sample was performed using DNA-free (Ambion, Huntingdon, Cambridgeshire, UK). cDNA was synthesised using 3'-RACE adaptor primer AP2 (AAGGATCCGTCGACATC(17)T), 5 µg total RNA and M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions. The polymerase chain reactions (PCRs) for the molecular cloning of the cDNA were done using the primer pairs Ex1up and Ex8lo (5' GCTCTTTTAAAGCTCCCCTGA 3'/5' CTGTCCAGTCCCAGAAGGAA 3') and primer pair Ex8up and 3'UTRlo (5' AGGGCATCTCGCAGGAGTC 3'/5' ATTCAAGTAACTGCAAATAGGA 3') which were derived from human cDNA sequences (accession no. X14957). The PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN, Hilden, Germany), cloned in pGEM-T Easy vector system (Promega, Madison, USA) and sequenced. The cDNA contig and the homology alignments were created with Lasergene software (DNASar, Madison, USA) and various sequences from the NCBI database (GenBank accession nos. X14957, X14958, NM\_002131, NM\_145899, NM\_145900, NM\_145901, NM\_145902, NM\_145903, NM\_145904, NM\_145905).

### 2.3. Characterisation of splice variants

The splice variants *HMGA1a* and *HMGA1b* were detected by amplifying a fragment spanning the CDS with primer pair Up (5' CATCCAGCCATCACTC 3') and Lo (5' GCGGCTGGTGTGCTGTGTAGTGTG 3') using the canine testis cDNA samples as template. The primer pair was designed using the cDNA cloned as described in Section 2.2. The obtained PCR products were separated on a 4.0% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM-T Easy vector system (Promega) and sequenced. The contigs and the homology alignments were created with two sequences from the NCBI database (GenBank accession nos. X14957, X14958).

#### 2.4. CDS comparison between breeds

The CDS of both splice variants were characterised for all breeds as described previously in Section 2.3. The contigs and the homology alignments were created using two sequences from the NCBI database (GenBank accession nos. X14957, X14958). In case of single nucleotide exchanges, the samples were sequenced again in both forward and reverse direction. Exchanges causing no amino acid (aa) substitution were not taken into account for further analyses. For all samples with aa substitutions the initial PCR was repeated and the exchange verified by sequencing the product in both forward and reverse direction. If possible, a restriction enzyme digestion was performed additionally.

#### 2.5. Protein sequences

The canine HMGA1a and HMGA1b protein sequences were derived from the open reading frames (ORFs) of the characterised cDNA sequences described previously in Section 2.2. The protein homology alignments were created with two sequences from the NCBI database (GenBank accession nos. X14957, X14958).

#### 2.6. Northern blot

Total RNAs were isolated from canine heart, lung, muscle, kidney and spleen tissue using RNeasy system (QIAGEN). An additional sample of total RNA was isolated from canine heart tissue by TRIZOL LS acid guanidine isothiocyanate–chloroform method (Invitrogen) in order to figure out whether this isolation method would lead to any difference in hybridisation. Further on poly A RNA was purified from canine spleen total RNA with OLIGOTEX (QIAGEN) and total RNA was prepared from human cultured fibroblasts by RNeasy system (QIAGEN). Spleen poly A RNA was placed on the blot in case that *HMGA1* was not detectable in the total RNA samples.

For Northern Blot hybridisation, 20 µg of total RNA from each sample with the exception of 10 µg of muscle and 3.6 µg of spleen poly A RNA were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto Hybond-N+ positive nylon membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary blot.

A 489-bp cDNA fragment derived from the canine HMGA1a sequence (exon 5/exon 8) served as a molecular probe for hybridisation. The probe was generated by PCR with the primer set Up and Lo (5' CATCCAGCCATCACTC 3'/5' GCGGCTGGTGTGCTGTGTAGTGTG 3') using the cloned cDNA described in Section 2.2. Probe labelling was performed by random primed labelling (Amersham Pharmacia Biotech) as described in the manufacturer's protocol with 50 µCi(α<sup>32</sup>P)dCTP (Amersham Pharmacia Biotech). Purification of the labelled probe was performed

using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at –20 °C before use.

Using the PERFECTHYB PLUS hybridisation solution (Sigma-Aldrich, Saint Louis, MO, USA) prehybridisation was carried out for 30 min and hybridisation for 2.5 h at 68 °C. The membrane was washed for 5 min at room temperature in 2×SSC/0.1% SDS, and twice for 20 min at 68 °C in 0.5×SSC/0.1% SDS. Signals were visualised using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, USA).

### 3. Results and discussion

#### 3.1. The canine *HMGA1* cDNA transcripts

For the human *HMGA1* gene various transcripts were described for both splicing variants (*HMGA1a* and *HMGA1b*) that differ in their 5'-UTR. The characterisation of the canine *HMGA1* cDNAs revealed that the complete canine *HMGA1* cDNA spans six exons and codes for two splicing variants *HMGA1a* with 1836 bp and *HMGA1b* with 1803 bp which are similar to the human transcripts (*HMGA1a* GenBank accession no. AY366390 and *HMGA1b* GenBank accession no. AY366392). The exon structure, the UTRs and the ORFs of both splice variants were defined and their homologies to their human counterparts analysed (Fig. 1, Table 1). The splicing variants showed the “typical” 33 bp gap which is conserved across various species such as human, mouse, hamster and rat (GenBank accession nos. BC013455, NM\_016660, A7193763, NM\_139327, A7511040). The homology of the canine cDNAs to their human counterparts is 80.6% for both splice variants. The 5'-UTR, CDS and the 3'-UTR showed homologies of 95.6%, 95.1% and 74.7%, respectively (Table 1). Homologies of the canine CDS with the CDS from mouse, hamster and rat on nucleotide level vary from 90.4% to 93.1%. The cDNA sequences were submitted to the NCBI database: *HMGA1a*, GenBank accession no. AY366390 and *HMGA1b*, GenBank accession no. AY366392.

#### 3.2. The canine *HMGA1a* and *HMGA1b* proteins

The canine HMGA1a and HMGA1b protein sequences were deduced from the respective cDNA sequences. The canine HMGA1a protein is a 107-amino acid molecule with a calculated weight of 11,674.97 D and HMGA1b a 96-amino acid molecule with a calculated weight of 10,677.85 D (Fig. 2). Homology comparison to the human counterparts (GenBank accession nos. P17096, X14957) showed 100% homology of the molecules including the three “AT-hooks” and the acidic carboxy-terminal domain.

Comparison of the canine and human HMGA1a and HMGA1b proteins with the described mouse, rat and hamster molecules showed aa changes in positions 5, 34,

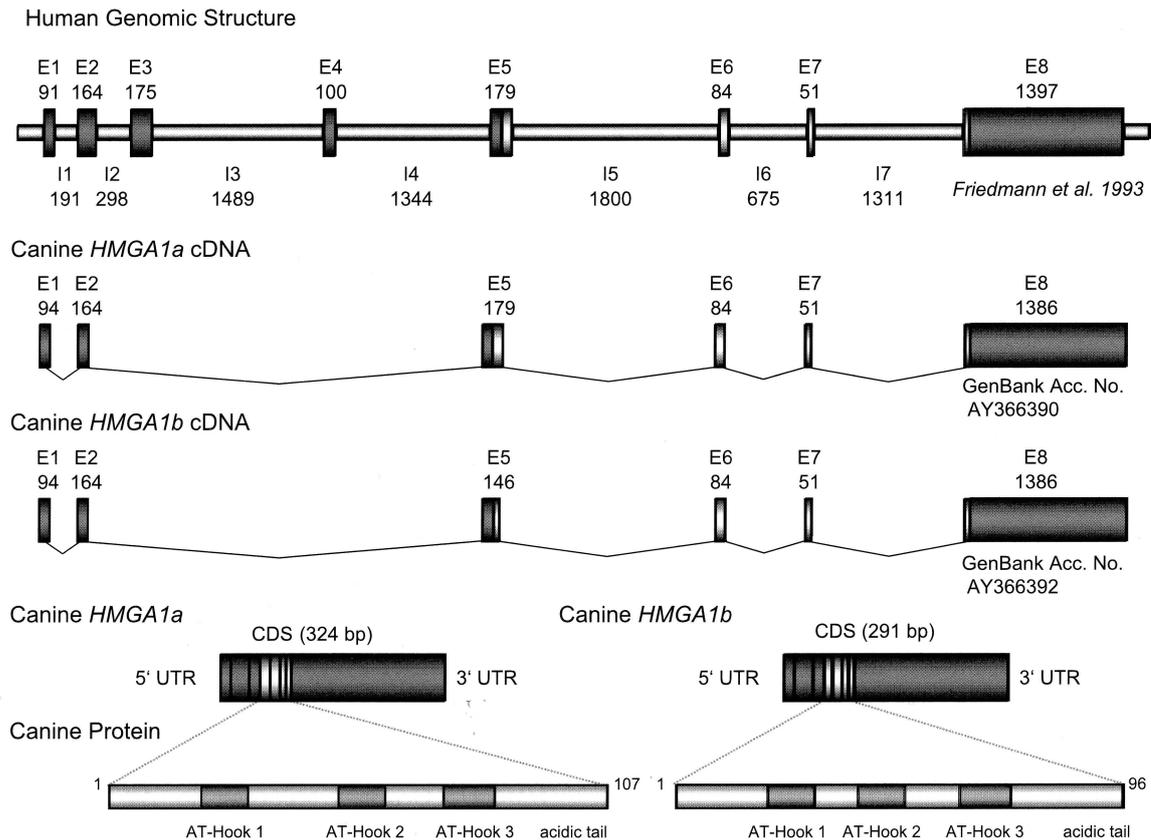


Fig. 1. Structure of the canine *HMGA1a* and *HMGA1b* transcripts and partial genomic structure.

69, 75 and 78 of *HMGA1a* and positions 5, 34, 58, 64 and 67 of *HMGA1b*, respectively (Fig. 2) (Johnson et al., 1988, 1989; Friedmann et al., 1993; Aldrich et al., 1999; Sgarra et al., 2000; Strausberg et al., 2002; Sgarra et al., 2003).

According to the definition of the AT-hooks (*HMGA1a*: I aa 21–31, II aa 53–63, III aa 78–89; *HMGA1b*: I aa 21–31, II aa 42–52, III aa 67–78) by Reeves and Nissen (1990) and Reeves (2000), none but the aa exchange at position 78

(*HMGA1a*) or 67 (*HMGA1b*), respectively, do affect the AT-hooks in either species. The exchange at position 78 leads to a difference in the third AT-hook of mouse and hamster when compared to the other species. According to the definition of the AT-hooks (*HMGA1a*: I aa 23–31, II aa 55–70, III aa 81–89; *HMGA1b*: I aa 23–31, II aa 44–59, III aa 70–78) by Huth et al. (1997), this aa exchange does not affect the third AT-hook. Following this definition, the second AT-hook is affected by the aa exchange at position 69 (*HMGA1a*) or 58 (*HMGA1b*), respectively.

The canine protein sequences were submitted to the NCBI database with GenBank accession nos. *HMGA1a* AY366390 and *HMGA1b* AY366392.

Due to the identical structure of the canine *HMGA* proteins to the respective human molecule, therapeutic approaches applied in dogs could be more suitable in terms of transferability for the development of human therapies than to approaches tested in other organisms.

### 3.3. *HMGA1a* and *HMGA1b* CDS comparison between canine breeds

For twelve different canine breeds the splicing variants *HMGA1a* and *HMGA1b* were detected by amplification of a fragment spanning the CDS using the canine testis cDNA samples as template. The comparison of the characterised protein coding sequences for these twelve canine breeds

Table 1

Detailed analysis of the canine *HMGA1a* and *HMGA1b* cDNA

Element of canine <i>HMGA1</i> cDNAs	Size in bp	Homology to human counterpart in %
Total cDNA <i>HMGA1a</i>	1836	80.6
Total cDNA <i>HMGA1b</i>	1803	80.6
5'-UTR	231	95.6
CDS <i>HMGA1a</i>	324	95.1
CDS <i>HMGA1b</i>	291	95.1
3'-UTR	1332	74.7
Exon 1	94	97.8
Exon 2	114	96.5
Exon 5 <i>HMGA1a</i>	179	93.9
Exon 5 <i>HMGA1b</i>	146	93.9
Exon 6	84	96.4
Exon 7	51	94.1
Exon 8	1386	75.4

Homology comparison of the cDNA elements of the canine *HMGA1* to its human counterpart (characterisation of the UTRs, the ORF and the exon sizes).



aggressiveness in some of these malignancies has led to the conclusion that *HMGA* expression may present a powerful prognostic molecular marker.

So far no studies analysing the *HMGA1* expression pattern in canine tumours have been carried out. Since these tumours occur spontaneously in dogs as well as in humans a canine *in vivo* analysing system could have significant value for research and drug development.

The causal role of *HMGA1* expression in the progression of carcinomas has been elucidated by a set of *in vitro* experiments involving *HMGA1* sense and antisense transfection assays (Wood et al., 2000a,b; Reeves et al., 2001). A proof of concept for a therapy aimed at the down-regulation of HMGA protein in tumours has been presented by Scala et al. (2000) who were able to show that an *HMGA1* antisense strategy using an adenoviral vector treatment of tumours induced in athymic mice caused a drastic reduction in tumour size.

Due to the similarities of human and canine tumours, the transfer of such experimental approaches could benefit cancer research in either species.

The comprehension of the canine *HMGA1* gene and its gene products could be the precondition for future new experimental approaches and for evaluating the canine gene product as potential target for therapeutic strategies using the dog as model system.

## References

- Abe, N., Watanabe, T., Sugiyama, M., Uchimura, H., Chiappetta, G., Fusco, A., Atomi, Y., 1999. Determination of high mobility group I(Y) expression level in colorectal neoplasias: a potential diagnostic marker. *Cancer Res.* 59, 1169–1174.
- Abe, N., Watanabe, T., Masaki, T., Mori, T., Sugiyama, M., Uchimura, H., Fujioka, Y., Chiappetta, G., Fusco, A., Atomi, Y., 2000. Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. *Cancer Res.* 60, 3117–3122.
- Aldrich, T.L., Reeves, R., Lee, C.C., Thomas, J.N., Morris, A.E., 1999. HMGI (Y) proteins implicated in amplification of CHO cell DNA. Unpublished, GenBank Accession No. AF193763.
- Bandiera, A., Bonifacio, D., Manfioletti, G., Mantovani, F., Rustighi, A., Zanconati, F., Fusco, A., Di Bonito, L., Giaccotti, V., 1998. Expression of HMGI (Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. *Cancer Res.* 58, 426–431.
- Becker, K., Escobar, H.M., Richter, A., Meyer, B., Nolte, I., Bullerdiek, J., 2003. The canine *HMGA1* gene maps to CFA 23. *Anim. Genet.* 34, 68–69.
- Chiappetta, G., Bandiera, A., Berlingieri, M.T., Visconti, R., Manfioletti, G., Battista, S., Martinez-Tello, F.J., Santoro, M., Giaccotti, V., Fusco, A., 1995. The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasias. *Oncogene* 10, 1307–1314.
- Chiappetta, G., Avantaggiato, V., Visconti, R., Fedele, M., Battista, S., Trapasso, F., Merciai, B.M., Fidanza, V., Giaccotti, V., Santoro, M., Simeone, A., Fusco, A., 1996. High level expression of the HMGI (Y) gene during embryonic development. *Oncogene* 13, 2439–2446.
- Chiappetta, G., Tallini, G., De Biasio, M.C., Manfioletti, G., Martinez-Tello, F.J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giaccotti, V., Fusco, A., 1998. Detection of high mobility group I HMGI (Y) protein in the diagnosis of thyroid tumors: HMGI (Y) expression represents a potential diagnostic indicator of carcinoma. *Cancer Res.* 58, 4193–4198.
- Fedele, M., Bandiera, A., Chiappetta, G., Battista, S., Viglietto, G., Manfioletti, G., Casamassimi, A., Santoro, M., Giaccotti, V., Fusco, A., 1996. Human colorectal carcinomas express high levels of high mobility group HMGI (Y) proteins. *Cancer Res.* 56, 1896–1901.
- Friedmann, M., Holth, L.T., Zoghbi, H.Y., Reeves, R., 1993. Organization, inducible-expression and chromosome localization of the human HMGI (Y) nonhistone protein gene. *Nucleic Acids Res.* 21, 4259–4267.
- Huth, J.R., Bewley, C.A., Nissen, M.S., Evans, J.N., Reeves, R., Gronenborn, A.M., Clore, G.M., 1997. The solution structure of an HMGI (Y)-DNA complex defines a new architectural minor groove binding motif. *Nat. Struct. Biol.* 4, 657–665.
- Johnson, K.R., Lehn, D.A., Elton, T.S., Barr, P.J., Reeves, R., 1988. Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMGI (Y). *J. Biol. Chem.* 263, 18338–18342.
- Johnson, K.R., Lehn, D.A., Reeves, R., 1989. Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMGI and HMGI-Y. *Mol. Cell. Biol.* 9, 2114–2123.
- Kazmierczak, B., Dal Cin, P., Wanschura, S., Borrmann, L., Fusco, A., Van den Berghe, H., Bullerdiek, J., 1998. HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. *Genes Chromosomes Cancer* 23, 279–285.
- Kingman, S., 2000. Painting a brighter future for dogs and humans. *Drug Discov. Today* 5, 127–128.
- Kuska, B., 1996. Sit, DNA, sit: cancer genetics going to the dogs. *J. Natl. Cancer Inst.* 91, 204–206.
- Ostrander, E.A., Galibert, F., Patterson, D.F., 2000. Canine genetics comes of age. *Trends Genet.* 16, 117–124.
- Reeves, R., 2000. Structure and function of the HMGI (Y) family of architectural transcription factors. *Environ. Health Perspect.* 108 (Suppl. 5), 803–809.
- Reeves, R., Beckerbauer, L., 2001. HMGI (Y) proteins: flexible regulators of transcription and chromatin structure. *Biochim. Biophys. Acta* 1519, 13–29.
- Reeves, R., Nissen, M.S., 1990. The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. *J. Biol. Chem.* 265, 8573–8582.
- Reeves, R., Edberg, D.D., Li, Y., 2001. Architectural transcription factor HMGI (Y) promotes tumor progression and mesenchymal transition of human epithelial cells. *Mol. Cell. Biol.* 21, 575–594.
- Scala, S., Portella, G., Fedele, M., Chiappetta, G., Fusco, A., 2000. Adenovirus-mediated suppression of HMGI (Y) protein synthesis as potential therapy of human malignant neoplasias. *Proc. Natl. Acad. Sci. U. S. A.* 97, 4256–4261.
- Sgarra, R., Diana, F., Bellarosa, C., Rustighi, A., Toller, M., Manfioletti, G., Giaccotti, V., 2000. Increase of HMGA1a protein methylation is a distinctive characteristic of tumor cells induced to apoptosis. Unpublished, GenBank Accession No. AF511040.
- Sgarra, R., Diana, F., Bellarosa, C., Dekleva, V., Rustighi, A., Toller, M., Manfioletti, G., Giaccotti, V., 2003. During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry. *Biochemistry* 42, 3575–3585.
- Strausberg, R.L., et al., 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U. S. A.* 99, 16899–16903.
- Tallini, G., Vanni, R., Manfioletti, G., Kazmierczak, B., Faa, G., Pauwels, P., Bullerdiek, J., Giaccotti, V., Van Den Berghe, H., Dal Cin, P., 2000. HMGI-C and HMGI (Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroid hamartomas, endometrial polyps, and uterine leiomyomas and is compatible with rearrangement of the HMGI-C and HMGI (Y) genes. *Lab. Invest.* 80, 359–369.
- Tamimi, Y., van der Poel, H.G., Denyn, M.M., Umbas, R., Karthaus, H.F., Debruyne, F.M., Schalken, J.A., 1993. Increased expression of high

- mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization. *Cancer Res.* 53, 5512–5516.
- Vail, D.M., MacEwen, E.G., 2000. Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest.* 18, 781–792.
- Williams, A.J., Powell, W.L., Collins, T., Morton, C.C., 1997. HMG1 (Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm. *Am. J. Pathol.* 150, 911–918.
- Wood, L.J., Maher, J.F., Bunton, T.E., Resar, L.M., 2000a. The oncogenic properties of the HMG-1 gene family. *Cancer Res.* 60, 4256–4261.
- Wood, L.J., Mukherjee, M., Dolde, C.E., Xu, Y., Maher, J.F., Bunton, T.E., Williams, J.B., Resar, L.M., 2000b. HMG-1/Y, a new c-Myc target gene and potential oncogene. *Mol. Cell. Biol.* 20, 5490–5502.

# **XI.**

## **DNA sequence, polymorphism, and mapping of luteinizing hormone receptor fragment (*LHCGR*) gene in Great Dane dogs.**

**Santos SE, Murua Escobar H, Sider LH, Winkler S, Aoki SM, Milazzotto MP, Campagnari F, Vannucchi CI, Bullerdiek J, Nolte I, Garcia JF.**

*Anim Genet. 2004; 35(1): 74-5.*

Contribution to the work

- Planning and coordination of the BAC screening
- Radioactive BAC screening
- Coordination of the international cooperation
- Composing of the publication together with Dr. S.E. Santos

small spherical to large spherical concepti, as found in a previous study<sup>3</sup>; however we could not confirm an increase in *Brachyury* expression from tubular to filamentous concepti (Fig. 1).

**Comments:** Our linkage mapping data places *PLET1* near *PPP2ARB* and *CRYAB*, but does not enable gene order to be determined. The human locus orthologous to *PLET1* is on chromosome 11q23.2 (LOC349633, LocusLink, NCBI), the evolutionary homologue of SSC9. In human, *PLET1*, *PPP2ARB*, and *CRYAB* are also tightly linked, with a gene order of *PPP2ARB* (*PPP2R1 beta*, 111,645 Kbp)-*CRYAB* (111,813 Kbp)-*PLET1* (LOC349633, 112,153 Kbp)-*APOA1* (116,740 Kbp). We also found the expression of *PLET1* increased during trophoblastic elongation in the porcine conceptus. The functional role of *PLET1* and factors that regulate its expression are currently unknown.

**Acknowledgements:** This work was supported by USDA NRI 99-3520S-8370. This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3600, was supported by Hatch Act and State of Iowa funds. We thank the collaborators for providing PiGMaP family DNA samples.

## References

- 1 Tuggle C. K. *et al.* (2003) *Mamm Genome* **14**, 565–79.
- 2 Archibald A. L. *et al.* (1996) *Mamm Genome* **6**, 157–75.
- 3 Yelich J. V. *et al.* (1997) *Biol Reprod* **57**, 1256–65.

**Correspondence:** Christopher K. Tuggle (cktuggle@iastate.edu)

doi:10.1111/j.1365-2052.2003.01080.x

## DNA sequence, polymorphism, and mapping of luteinizing hormone receptor fragment (*LHCGR*) gene in Great Dane dogs

**S. E. C. Santos\***, **H. Murua Escobar<sup>†,§</sup>**, **L. H. Sider\***, **S. Winkler<sup>‡</sup>**, **S. M. Aoki\***, **M. P. Milazzotto\***, **F. Campagnari\***, **C. I. Vannucchi<sup>†</sup>**, **J. Bullerdiek<sup>‡</sup>**, **I. Nolte<sup>§</sup>** and **J. F. Garcia\***

\*Department of Animal Health and Production, São Paulo State University, Araçatuba, Brazil. <sup>†</sup>Department of Animal Reproduction, University of São Paulo, São Paulo, Brazil. <sup>‡</sup>Center for Human Genetics, University of Bremen, Bremen, Germany. <sup>§</sup>Clinic for Small Animal, School of Veterinary Medicine of Hanover, Hanover, Germany

Accepted for publication 29 November 2003

**Source/description:** Exon 11 of the canine luteinizing hormone receptor (*LHCGR*) gene (960 bp) was polymerase chain reaction (PCR) amplified and sequenced. Primers were designed based upon previous bovine *LHCGR* gene sequence (Genbank: 420504). Activating and inactivating mutations have been described in the same receptor in humans.<sup>1</sup>

**DNA isolation:** Genomic DNA was isolated from Great Dane dogs peripheral blood leucocytes using phenol/chloroform purification based protocols.<sup>2</sup>

## Primers sequences:

Primer 1: 5' TTATTCTGCCATCTTTGCTGAGA 3'

Primer 2: 5' CTCAGCAACAAAAGAAATCCT 3'

Genbank accession number: AF389885

**PCR conditions:** The PCR amplifications were performed in a final volume of 50 µl containing DNA (10 ng), dNTPs 1.25 mM each, *Taq* DNA polymerase (2 UI), *Taq* DNA polymerase buffer 10X, magnesium chloride (2.5 mM) and ultra pure water. Amplification parameters were: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 30 s, 72 °C for 2 min and one final extension step of 72 °C for 8 min. Negative controls were performed adding all reagents except DNA. All PCR fragments were subsequently submitted to automatic DNA sequencing.

**LHCGR:** The canine exon 11 fragment shared 89, 89, 92 and 88% identity to the human, bovine, porcine and murine *LHCGR* sequences, respectively.

**Sequence analysis:** Sequencing of PCR fragments was performed on the ABI 310 (Applied Biosystems®, Norwalk, CT)

**Polymorphism:** Two polymorphic sites were identified in codons encoding amino acid positions Ile 378 and Val 397 of *LHCGR*. Both polymorphism were silent mutations.

**LHCGR isolation of BAC clone containing LHR gene:** A canine genomic DNA probe was used for hybridization of canine RPCI 81 BAC/PAC filter (Bacpac Resources/Children's Hospital Oakland Research Institute, Oakland, USA). The 570 bp probe was generated by PCR (Primer 5' TTATTCTGCCATCTTTGCTGAGA 3'/5' CCTCCGAGCATGACTGGAATGGC 3') on genomic DNA of the canine pleomorphic adenoma cell line ZMTH3 (Center for Human Genetics, Bremen, Germany). The obtained PCR product was separated on a 1.5% agarose gel, recovered with QIAEX II (Qiagen, Leusden, Netherlands), cloned in pGEM T-easy vector system (Promega, Madison, USA) and sequenced for verification. The probe labelling was performed by random primed labelling (Roche Diagnostics, Mannheim, Germany) as described in the manufacturer's protocol with 50 µCi( $\alpha$ <sup>32</sup>P)dCTP (Amersham Pharmacia Biotech, Freiburg, Germany). Purification of the labelled probe was carried out using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at -20 °C before use. The filters were placed in a minimum volume of Church Buffer (0.15 mM bovine serum albumin, 1 mM ethylenediaminetetraacetic acid, 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulphate) and transferred into hybridization bottles. The filters were pre-hybridized at 65 °C for 1 h in 25 ml Church Buffer. Hybridization was performed at 65 °C overnight (16–18 h) in the same solution. All further steps were performed according to manufacturer's protocol. Signals were visualized using a STORM imager (Molecular Dynamics, Sunnyvale, USA).

**Fluorescence in situ hybridization:** Metaphase preparations were obtained from blood samples of two different dogs. The samples were stimulated with PHA and cultured for 96 h at 37 °C. After a 1.5 h colcemide (0.1 µg/ml) incubation, the lymphocytes were harvested according to routine procedures. Prior to fluorescence *in situ* hybridization (FISH), chromosomes were stained using the GTG-banding method. After obtaining the

metaphases, the slides were destained in 70% ethanol for 15 min and air dried. FISH was performed using the protocol with some modifications.<sup>3</sup> BAC DNA (RPC181 272N8) was digoxigenin labelled (Dig-Nick-Translation-Kit; Roche Diagnostics). The hybridization mixture contained 125–175 ng probe, 43.2 µg salmon sperm DNA, 1000–1200 ng sonicated dog DNA, 2× SSC, 2× SSPE, 50% formamide and 10% dextran sulphate. The chromosomes were stained with propidium iodide. Chromosomal G-bands were identified.<sup>4</sup> Canine *LHCGR* was located on chromosome CFA 10.

*Acknowledgements:* FAPESP – São Paulo – Brasil.

## References

- 1 Themmen A.P. *et al.* (1998) *Molecular and Cellular Endocrinology* **145**, 137–142.
- 2 Sambrook J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- 3 Fischer P. *et al.* (1996) *Mammalian Genome* **7**, 37–41.
- 4 Reimann N. *et al.* (1996) *Cytogenetics Cell Genetics* **73**, 140–144.

*Correspondence:* S. E. C. Santos (silviacrusco@terra.com.br)

doi:10.1111/j.1365-2052.2003.01081.x

## Identification of two polymorphic microsatellites in a canine BAC clone harbouring a putative canine *MAOA* gene

J. Klukowska\*, I. Szczerbal<sup>†</sup>, A. Wengi-Piasecka<sup>‡</sup>, M. Switonski<sup>†</sup>, C. Schelling<sup>‡</sup>, A. Gmür\* and G. Dolf\*

\*Institute of Animal Genetics, Nutrition and Housing, University of Berne, Switzerland. <sup>†</sup>Department of Animal Genetics and Breeding, August Cieszkowski Agricultural University of Poznan, Poland. <sup>‡</sup>Department of Animal Sciences, Swiss Federal Institute of Technology of Zurich and Faculty of Veterinary Medicine and University of Zurich, Switzerland

Accepted for publication 29 November 2003

*Source/description:* Monoamine oxidase A (*MAOA*) is a mitochondrial enzyme which degrades the neurotransmitters noradrenaline and serotonin. Pharmacological and genetic studies suggest that variations in the sequences of the *MAOA*

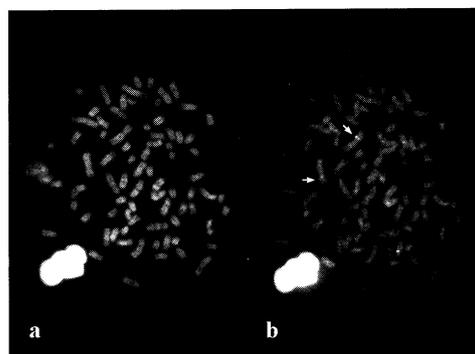
gene could be associated with behavioural changes including aggression and cognitive dysfunction<sup>1</sup>. A canine bacterial artificial chromosome (BAC) library<sup>2</sup> (<http://www.dogmap.ch/getclone.htm/>) was screened by polymerase chain reaction (PCR) using primers designed to amplify part of the canine *MAOA* exon 15 (AB038563). Subcloning of S048P05H11, subsequent screening and primer design (OLIGO 6.63; Med Probe, Oslo, Norway) yielded the two microsatellites *ZuBeCa57* (F: 5'-ATCTGAGGTCCTGGTACTGAATC, R: 5'-GTGAAAAA TTAAGTCAAGGTAGGT) and *ZuBeCa61* (F: 5'-GGCTGCTT GGGGGATTTCTGCTTAAT, R: 5'-TTGTAGAGGACCAGGACG CGCACAC).

*PCR conditions:* Amplifications were carried out in 12 µl containing 2 µl of DNA solution, 2.5 pmol of each primer, 0.25 mM of each dNTP, 1X PCR buffer with 1.5 mM MgCl<sub>2</sub> and 0.35 units *Taq* polymerase (Qbiogene, Basel, Switzerland) in a Perkin-Elmer 9700 thermocycler. PCR was performed using the following touch-down programme<sup>3</sup>: initial denaturation for 5 min at 94 °C, two cycles each of 30 s at 94 °C, 30 s in the respective touch-down range down from the highest to the lowest annealing temperature (63–55 °C) and 30 s at 72 °C, followed by six cycles of 30 s at the lowest annealing temperature. The final extension was for 15 min at 72 °C. Sizes of the alleles were determined on 8% denaturing polyacrylamide gels using a LI-COR DNA sequencer model 4200 (LI-COR, Bad Homburg, Germany).

*Nucleotide sequence:* GeneBank accession numbers:

*ZuBeCa57:* AJ604537

*ZuBeCa61:* AJ604538



**Figure 1** Q-banded metaphase spread of a female dog (a) and the same spread after FISH with the BAC SP048P05H11 (b). The arrows indicate the localization on CFAx11.

**Table 1** Summary data for two canine microsatellites.

	<i>ZuBeCa57</i> (TAAA) <sub>11</sub>				<i>ZuBeCa61</i> (GT) <sub>13</sub>			
	Size range	No. of alleles	PIC	HET	Size range	No. of alleles	PIC	HET
Dog ( <i>n</i> = 24)	207–223	5	0.49	0.57	83–103	3	0.19	0.21
Red fox ( <i>n</i> = 10)	203–211	3	0.56	0.71	85–89	3	0.43	0.57
Arctic fox ( <i>n</i> = 10)	203–215	3	0.18	0.21	85–101	5	0.66	0.78
Chinese raccoon dog ( <i>n</i> = 10)	199–207	3	0.41	0.54	93–111	4	0.48	0.58

## **XII.**

**A 3.4-kbp transcript of *ZNF331* is solely expressed in  
follicular thyroid adenomas.**

**Meiboom M, Murua Escobar H, Pentimalli F, Fusco A, Belge G,  
Bullerdiek J.**

*Cytogenet Genome Res. 2003; 101(2): 113-7.*

Contribution to the work

- Radioactive probe labelling
- Northern Blot Hybridisation
- Blot imaging

## A 3.4-kbp transcript of ZNF331 is solely expressed in follicular thyroid adenomas

M. Meiboom,<sup>a</sup> H. Murua Escobar,<sup>a</sup> F. Pentimalli,<sup>b</sup> A. Fusco,<sup>b</sup> G. Belge<sup>a</sup> and J. Bullerdiek<sup>a</sup>

<sup>a</sup>Center for Human Genetics, University of Bremen, Bremen (Germany);

<sup>b</sup>Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università di Napoli 'Federico II', Naples (Italy)

**Abstract.** Translocations involving chromosomal region 19q13 are a frequent finding in follicular adenomas of the thyroid and might represent the most frequent type of structural aberration in human epithelial tumors. By positional cloning, a putative candidate gene, ZNF331 (formerly RITA) located close to the breakpoint was identified. Recently, aberrant expression of ZNF331 has been described in two cell lines of follicular thyroid adenomas with aberrations in 19q13 indicating an involvement of ZNF331 in tumorigenesis. Nevertheless, knowledge about structure and expression of ZNF331 is limited.

We performed RACE-PCR and genomic sequence analyses to gain a deeper insight into its molecular structure. To elucidate ZNF331 expression patterns we performed Northern blot analyses on various normal tissues as well as on thyroid carcinoma and adenoma cell lines. Herein, unique expression of a 3.4-kbp transcript is described in thyroid adenoma cell lines with 19q13 aberrations, which was not detected either in normal tissues or in thyroid carcinoma cell lines.

Copyright © 2003 S. Karger AG, Basel

By positional cloning of the 19q13 breakpoints found in follicular adenomas of the thyroid gland, we had recently been able to identify a candidate gene (Rippe et al., 1999) which we primarily referred to as RITA (Rearranged in Thyroid Adenomas) and which was renamed ZNF331 according to the HUGO human gene nomenclature. ZNF331 is considered a candidate gene relevant for development of thyroid adenomas due to the fact that aberrations of chromosomal band 19q13.4 are the most frequent structural chromosomal alterations in thyroid follicular adenomas and hyperplasias (Belge et al., 1998). ZNF331 was characterized as a KRAB zinc finger protein and is therefore most likely considered to be a transcriptional repressor. Molecularly, so far only about 2 kbp of cDNA

sequence, including the open reading frame, from ZNF331 have been characterized. The cDNA encodes a 463 amino acid protein consisting of a 42-aa KRAB-A domain and a 332-aa DNA-binding zinc finger region separated by an 89-aa spacer region (Rippe et al., 1999; Wu et al., 2001). In contrast to the results of Wu et al. (2001) who found expression of ZNF331 (or here called ZNF463) only in testis as an approximately 2.1-kbp transcript, we found expression of ZNF331 also in Northern blot analyses from normal thyroid, myometrium, testis tissues, and two cell lines from follicular thyroid adenomas with aberration in chromosomal band 19q13.4 (Rippe et al., 1999). Two transcripts of about 4.0 and 4.8 kbp were seen in each of the normal tissues tested. In addition, expression of a 2.1-kbp transcript was seen in testis only. Each of the cell lines from thyroid adenomas showed expression of two transcripts of approximately 5.5 and 6.2 kbp.

To get further information on expression profiles we tested various normal tissues as well as cell lines of various thyroid carcinoma cell lines for expression of ZNF331. Northern blot hybridizations were performed with a probe representing part of the 5'UTR and parts of the gene encoding the KRAB domain, spacer region, and part of the zinc finger domain.

Supported by the Deutsche Forschungsgemeinschaft.

Received 2 June 2003; manuscript accepted 11 July 2003.

Request reprints from Dr. J. Bullerdiek, Center for Human Genetics  
University of Bremen, Leobener Str. ZHG, DE-28359 Bremen (Germany)  
telephone: 49-421-218-4239; fax: 49-421-218-4239  
e-mail: bullerd@uni-bremen.de

Although sequence alignments of this probe with sequences from the NCBI database did not show homologies critical for hybridization, we performed additional Northern blot hybridizations with a ZNF331-specific probe derived from a part of the gene presenting neither the zinc finger region nor the KRAB domain to overcome potential cross-hybridizations with transcripts of other zinc finger genes.

In an effort to characterize the larger transcripts detected in Northern blot hybridization we performed RACE experiments on cDNA from normal thyroid and testis tissue and from material of a cell line of a thyroid adenoma. The results of these studies also enabled us to establish part of the genomic structure of ZNF331. Here we present molecular data of novel splice variants of ZNF331 and of the genomic sequence of this gene as well as novel data of its expression patterns in various tissues.

## Materials and methods

### *Tissue samples and cell lines*

Tissue samples were frozen in liquid nitrogen immediately after surgery and stored at  $-80^{\circ}\text{C}$ . The human thyroid carcinoma cell lines used in these studies were TPC1, NIM1, NPA, FRO and ARO (Pang et al, 1989; Fagin et al., 1993; Zeki et al., 1993; Cerutti et al., 1996; Cassoni et al., 2002). Thyroid adenoma cell lines with t(5;19)(q13;q13) and t(1;19)(p35 or p36.1;q13), respectively, were obtained by transfecting primary cells of a thyroid adenoma and a thyroid goiter, respectively, with a construct containing the SV40 large T-antigen, as reported previously (Belge et al., 1992).

### *RNA isolation and Northern blot analysis*

Poly(A)<sup>+</sup> RNAs were purified by Oligotex dC<sub>10</sub>T<sub>30</sub> adsorption (QIAGEN, Hilden, Germany) or oligo (dT) cellulose (Roche, Monza, Italy) from five thyroid carcinoma cell lines, two thyroid adenoma cell lines, normal fibroblasts, and normal adult tissues. Approximately 5 µg of poly(A)<sup>+</sup> RNAs were denatured and fractionated on a 1% agarose, 6% formaldehyde gel, and transferred onto a Hybond-N<sup>+</sup> nylon membrane (Amersham Pharmacia, Freiburg, Germany). Additionally, expression patterns of ZNF331 in normal tissues were analysed by using the human MTN IV (multiple tissue Northern blot IV) membrane (Clontech, Palo Alto). As molecular probes we used partial cDNA clones of ZNF331 i.e. one cDNA clone representing the spacer region and one cDNA clone containing the KRAB domain, spacer region and zinc finger domain. Probes were generated either by *EcoRI* digestion (174 bp fragment, spacer region) or by PCR with primers ACG CAA CCG CTG TGT CTC C and AGG CCT TCC CAC ATT CTT GAC (1,092 bp, KRAB domain, spacer region, zinc finger domain). Probes were labeled with <sup>32</sup>P using a random primer extension protocol (Feinberg and Vogelstein, 1983). Signals were visualized using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, USA).

### *cDNA synthesis*

For cDNA synthesis 5 µg of total RNA from normal testis and thyroid tissue as well as from cell line S40.2T/SV40 was reverse transcribed into first strand cDNA by SuperScript<sup>TM</sup> Reverse Transcriptase (Life Technologies, Karlsruhe, Germany) following the manufacturers instructions. For use in 5' RACE experiments two downstream gene specific primers, TCG GCG AAC GTC ACC AAA and CCT TCC CAC AGT CCT TAC ATT CAA, were used as start for the transcription. cDNA was tailed with dCTP creating a binding site for 5' anchor primer CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG.

### *Rapid amplification of cDNA ends*

For 5' RACE experiments dC-tailed cDNA was amplified using 400 nM of 5' anchor primer with either primer CAU CAU CAU CAU TCG GCG AAC GTC ACC AAA or primer CAU CAU CAU CAU CTC TGG AGC GCT GTG GTC TTT CAA. Alternatively, the adaptor-ligated double-stranded Marathon-Ready cDNA library from human testis (Clontech Laboratories, Palo Alto, CA) was used as template. 5 µl of the library were used for

amplification with the upstream adaptor primer AP1 (CCATCCTAATAC-GACTCACTATAGGGC) (Clontech Laboratories, Palo Alto, USA) and a gene specific downstream primer (TCA CAT ATC CAG TTA CGG CCA AGG GAT TTA CTT C) in a touchdown PCR reaction. PCR products were separated in a 1.5% agarose gel and recovered by glass bead techniques (QIAexII Gel Isolation Kit, QIAGEN, Hilden, Germany), cloned into the pGEM-T Easy (Promega, Mannheim, Germany) or into the pAMP1 vector system (Gibco BRL, Life Technologies, Eggenstein, Germany) and subjected to DNA sequence analysis.

### *DNA sequence analysis*

DNA sequence analysis and primer design were performed with the Lasergene software package (DNASTar, Madison, USA). Analyses for sequence homologies were performed using the BLAST program from NCBI (Altschul et al., 1997). Accession numbers for ZNF331: AF272148; ZNF331 splice variants: AF412398; AF412399; AF412400; AF412401; AF412402; AF412403; AF412404; BF057584; BC009433; AF251515; BF685522.

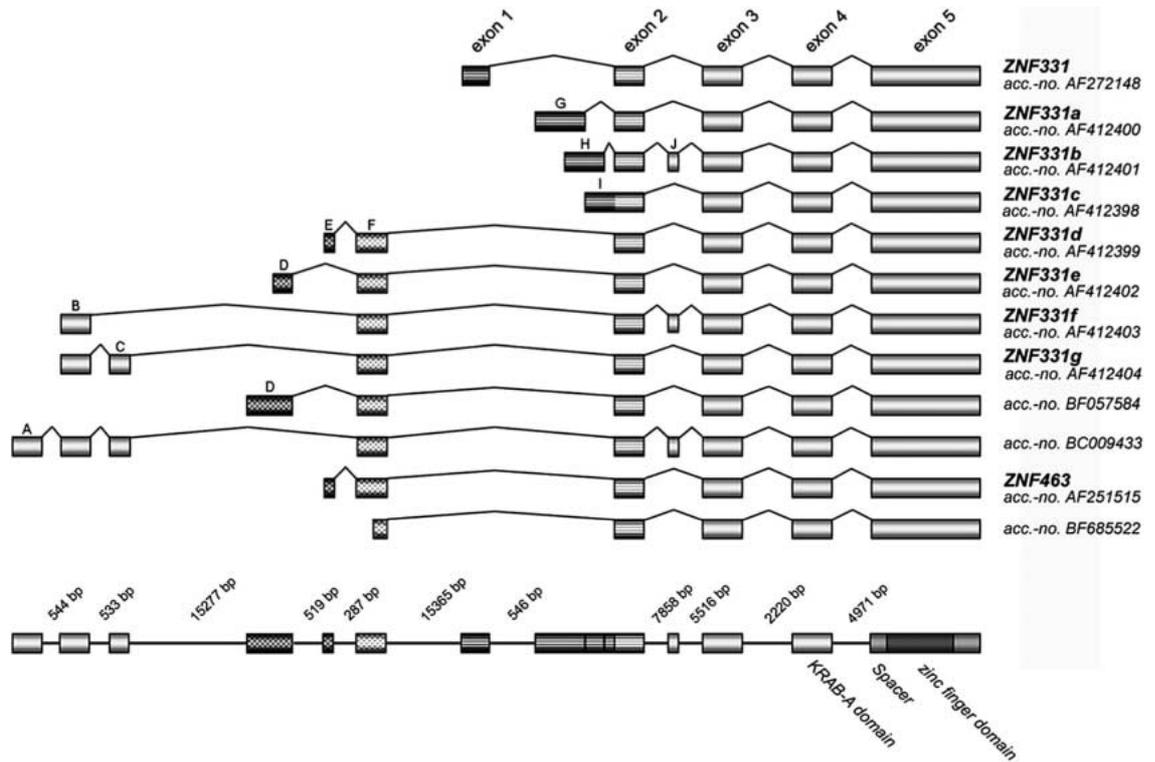
## Results

### *RACE*

Sequencing of 5' RACE products revealed seven new splice variants of ZNF331 (Fig. 1). In each investigated clone the exons 5, 4, and 3 representing the zinc finger region, the KRAB domain and the part of 5' UTR including the ATG start codon were identical. Differences in cDNA sequence were upstream of exon 2 except for two clones, i.e. ZNF331b and ZNF331f, which had an additional 30-bp insertion between exon 2 and 3. Clone ZNF331c revealed an extended exon 2, clones ZNF331d, e, f, and g contained a 67-bp exon upstream of exon 2, which differs by only 4 bp from exon 2. Clones ZNF331d, e, f, and g containing the duplicated exon 2 revealed sequence homology with ESTs from the NCBI database. All sequences correspond to the known genomic sequence.

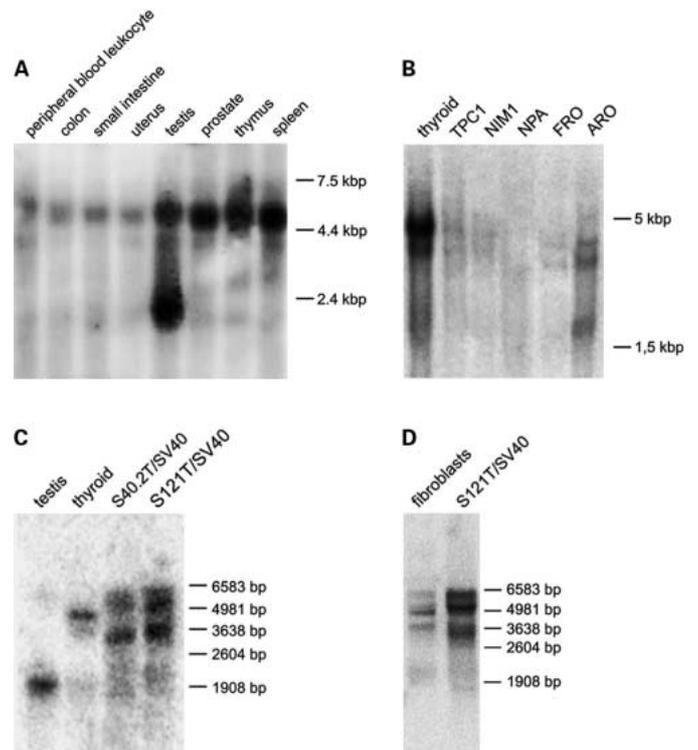
### *Northern blot hybridization*

Northern blot hybridization of a ZNF331-specific probe containing the KRAB domain, spacer region and zinc finger domain on poly(A)<sup>+</sup> RNA of several normal tissues (human MTN IV membrane, Clontech) revealed a transcript of about 4.8 kbp in all tissues. A much fainter signal is seen at about 4.0 kbp. In testis, an additional 2.1-kbp transcript is clearly seen (Fig. 2A). Hybridization of the same probe on poly(A)<sup>+</sup> RNA from thyroid carcinoma cell lines and normal thyroid tissue revealed two transcripts of about 4.0 and 4.8 kbp in normal thyroid tissue and cell lines TPC1, NIM1, FRO and ARO. In thyroid tissue, the expression of the 4.8-kbp transcript is much stronger than in the cell lines. In ARO, an additional transcript is seen at about 2.1 kbp corresponding to the band seen in testis. Cell line NPA did not show expression of ZNF331 (Fig. 2B). Hybridization of a probe derived from the spacer region of ZNF331 (174 bp, *EcoRI* fragment) on poly(A)<sup>+</sup> Northern blots revealed two signals of approximately 4.0 and 4.8 kbp in normal thyroid tissue and normal fibroblasts (Fig. 2C and 2D). In testis RNA, a strong band of 2.1 kbp was detected. None of these transcripts was found in the two cell lines with 19q aberrations, which instead expressed three distinct transcripts of about 3.4, 5.5 and 6.2 kbp (Fig. 2C). Hybridization of this probe on normal fibroblasts showed additional weak signals at 5.5 and 6.2 kbp which were not detected by



**Fig. 1.** Schematic genomic organization of ZNF331. Splice variants of ZNF331, three representative ESTs from the databases and ZNF463 (Wu et al., 2001) are aligned above. For a clearer presentation we indicated the newly found exons by letters and kept the numerical indication for the known exons (Rippe et al., 1999) and exon 5 has been graphically given the same size although EST sequences did not fully cover this region. Squares and lines, respectively mark exons lying in duplicated regions. The nearly identical exons 2 and F, representing the starting points of the duplicated regions, are presented in a lighter tone.

**Fig. 2.** Expression studies of ZNF331. **(A)** Northern blot analysis of poly(A)<sup>+</sup> RNA from various human tissues (MTN IV, Clontech, Palo Alto). An expression of a 4.8-kbp transcript is seen in all tissues. Most tissues also show a weaker expression of an approximately 4.0-kbp transcript. In testicular tissue, an additional band of 2.1 kbp is seen. **(B)** Northern blot analysis of poly(A)<sup>+</sup> RNA from normal thyroid tissue and thyroid carcinoma cell lines. Two transcripts of about 4.0 and 4.8 kbp are seen in normal thyroid tissue and cell lines TPC1, NIM1, FRO and ARO (lanes 2, 3, 5, 6). In ARO, an additional transcript is seen at about 2.1 kbp. Cell line NPA (lane 4) shows no expression of ZNF331. Compared to normal thyroid tissue (lane 1) the expression of these transcripts is strongly reduced. The gel shift occurring here was adjusted by consideration of ribosomal bands. **(C)** Northern blot analysis of poly(A)<sup>+</sup> RNA isolated from testis, normal thyroid tissue, cell line S40.2T/SV40, and cell line S121T/SV40 revealing two transcripts of about 4.0 and 4.8 kbp, respectively, of different intensity in thyroid (lane 2). An additional transcript of about 2.1 kbp is found exclusively in testis (lane 1). Cell lines S40.2T/SV40 and S121T/SV40 (lanes 4, 5) express three distinct transcripts of approximately 3.4, 5.5 and 6.2 kbp, respectively. **(D)** Comparative Northern blot analysis of poly(A)<sup>+</sup> RNA isolated from cultured normal fibroblasts (lane 1) and cell line S121T/SV40 (lane 2) with fibroblasts showing two transcripts of 4.0 and 4.8 kbp and additional weak signals at 5.5 and 6.2 kbp and cell line S121T/SV40 expressing three transcripts of 3.4, 5.5 and 6.2 kbp.



using a probe containing the KRAB domain, spacer region, and zinc finger domain of ZNF331 (data not shown). The 3.4-kbp transcript was not detected in normal fibroblasts with either of the probes and was thus solely found to be expressed in the cell lines with 19q13 aberrations.

#### *Genomic characterization*

Alignment of cDNA sequences with genomic DNA allowed the prediction of three transcription start sites for ZNF331 splice variants. Transcription start sites for ZNF331 and ZNF331a, b, and c (Fig. 1) are supposed to be upstream of exon 1 indicating that these splice variants span about 23 kbp on the genomic level. Further transcription start sites are most likely about 16 kbp (ZNF331d, e) and 32 kbp (ZNF331f, g) upstream the transcription start point for ZNF331 and ZNF331a, b, and c. Taking this into account, at the genomic level ZNF331 spans approximately 56 kbp.

#### **Discussion**

During the past ten years, the number of genes known to be affected by recurrent chromosomal translocations in human solid tumors has continuously increased. Interestingly, most of these gene rearrangements have been found in tumors of mesenchymal origin, whereas only a few specific chromosomal translocations have been described in epithelial tumors. Accordingly, the number of genes known to be affected by the latter aberrations is still low. The 19q13 translocations found in a cytogenetic subgroup of thyroid follicular adenomas represent one of the most frequent types of specific translocations in human epithelial tumors if not even representing the most frequent translocation at all. Chromosome 19 appears to be particularly enriched for zinc finger genes with ZNF loci distributed within three gene clusters corresponding to chromosomal bands p12, q13.2 and q13.4 (Bellefroid et al., 1993; Shannon et al., 1996). In KRAB zinc finger genes the KRAB domain recently has been shown to function as a critical domain for protein-protein interaction (Friedman et al., 1996; Kim et al., 1996). Although these genes may theoretically be involved in transcriptional activation or repression, all KRAB zinc finger genes studied so far have been shown to act only as transcriptional repressors (Margolin et al., 1994; Witzgall et al., 1994; Vissing et al., 1995). Accordingly, engineered chimeric fusion proteins consisting of KRAB domains combined with different DNA binding domains of oncogenic transcription factors have been shown to target their respective oncogene and specifically inhibit malignant growth and suppress tumorigenesis (Fredericks et al., 2000; Nawrath et al., 2000). As altered expression of such strong transcriptional repressors might well be the cause of tumor development, we have put effort not only to elucidate further the genomic structure of ZNF331 but also to extend our knowledge about the expression patterns of ZNF331.

In various normal tissues, ZNF331 is expressed as two transcripts of 4.0 and 4.8 kbp. An additional transcript of about 2.1 kbp is seen only in testis tissue (Fig. 2A). In papillary and anaplastic thyroid carcinoma cell lines TPC1, NIM1, FRO, and ARO, expression of the 4.0- and 4.8-kbp transcripts is seen.

However, compared to normal thyroid tissue, the expression of these transcripts is strongly reduced. The anaplastic carcinoma cell line ARO showed an additional transcript of about 2.1 kbp as it is also seen in normal testis tissue. Cell line NPA showed no expression of ZNF331 (Fig. 2B). These results are in contrast to the findings in follicular thyroid adenoma cell lines with 19q13.4 alterations. Here expression of three transcripts of about 3.4, 5.5 and 6.2 kbp is seen (Fig. 2C) (Rippe et al., 1999). However, the 5.5- and 6.2-kbp transcripts are also expressed at a very low level in cultured cells of normal fibroblasts (Fig. 2D), which was only detected by using a probe of ZNF331 containing only the spacer region. These spacer regions are known to be highly variable and are often used to distinguish between different members of the KRAB zinc finger gene family (Bellefroid et al., 1993). Apparently, the spacer probe was more sensitive than the probe containing the KRAB domain, spacer region, and zinc finger domain because the latter probe gave no signal at 5.5 and 6.2 kbp when applied to the fibroblast Northern blot. Thus, only the 3.4-kbp transcript is solely expressed in follicular thyroid adenomas with the 19q13.4 aberration. The correlation of expression of the 5.5- and 6.2-kbp transcripts and chromosome 19q13.4 aberration must be reconsidered, as these transcripts are also found in normal cultured fibroblasts pointing to an influence of cell culturing on the expression profile. However, in thyroid adenoma cell lines, expression of these transcripts is significantly stronger. In thyroid carcinoma cell lines, ZNF331 transcripts differing from those found in the normal tissues are not found. Here, expression of ZNF331 is strongly reduced compared to normal thyroid tissue with the molecular background behind this alteration still remaining to be elucidated. Considering the different transcript sizes of ZNF331 in follicular thyroid adenoma cell lines with 19q aberrations and thyroid carcinoma cell lines or normal tissues, respectively, it could be deduced that expression of the 3.4-kbp transcript is strongly associated with tumor type and involvement of chromosome 19q13.4 alteration.

By performing RACE experiments, we found seven different cDNAs coding for ZNF331. For a clearer presentation of the results, we used letters to name the newly found exons and kept the numerical indication of the exons described by Rippe et al. (1999). The differences in the new cDNAs were found to be only in the 5' UTR. 3' RACE experiments as well as database research confirmed the previously described 3' end of ZNF331 (Rippe et al., 1999). Four of these new cDNAs, i.e. ZNF331d, e, f and g, contained a new exon (F) which is nearly identical to the previously described exon 2. Genomic characterization showed this 67-bp exon F lying about 17 kbp upstream of exon 2. The genomic region surrounding exon F with about 660 bp has a 93% homology to the region surrounding exon 2. Further sequence analyses of the genomic region surrounding exon F showed a segment of about 1,300 bp to have high homology to the corresponding region, i.e. exon 1, intron 1 and exon 2 of ZNF331. An overall homology of about 90% is found between the two regions with a sequence segment of about 500 bp lacking in the region surrounding exon F. It has been discussed that the clustered localization of zinc finger proteins results from duplication of genomic segments which was supported by various repeat sequences lying in these regions i.e.

pericentromeric and telomeric regions (Eichler et al., 1998). Sequence homology of ZNF331 d, e, f and g with ESTs from the databases strongly indicates that these mRNAs represent relevant transcripts.

In summary, we have characterized most of the genomic structure of ZNF331. This KRAB zinger finger gene lies in close vicinity to a frequent breakpoint region of follicular adenomas. In thyroid adenoma cell lines with alterations in 19q13.4 expression studies revealed expression patterns different from those

seen in normal tissues, normal cultured cells, and carcinoma cell lines. This is most likely the result of the structural aberration in 19q13.4 altering the transcription of ZNF331. The unique expression of a 3.4-kbp transcript of ZNF331 in the tumor cell with 19q13 alterations strongly supports the hypothesis that ZNF331 is involved in the development of follicular adenomas. The molecular characterization of the 3.4 kbp transcript as well as a characterization of the promoter and functional analyses remain to be performed in future studies.

## References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Acids Res* 25:3389–3402 (1997).
- Belge G, Kazmierczak B, Meyer-Bolte K, Bartnitzke S, Bullerdiek J: Expression of SV40 T-antigen in lipoma cells with a chromosomal translocation t(3;12) is not sufficient for direct immortalization. *Cell Biol Int Rep* 16:339–347 (1992).
- Belge B, Roque L, Soares J, Bruckmann S, Thode B, Fonseca E, Clode A, Bartnitzke S, Castedo S, Bullerdiek J: Cytogenetic investigations of 340 thyroid hyperplasias and adenomas revealing correlations between cytogenetic findings and histology. *Cancer Genet Cytogenet* 101:42–48 (1998).
- Bellefroid EJ, Marine JC, Ried T, Lecocq PJ, Riviere M, Amemiya C, Poncelet DA, Coulie PG, de Jong P, Szpirer C: Clustered organization of homologous KRAB zinc-finger genes with enhanced expression in human T lymphoid cells. *EMBO J* 12:1363–1374 (1993).
- Cassoni P, Muccioli G, Marrocco T, Volante M, Allia E, Ghigo E, Deghenghi R, Papotti M: Cortistatin-14 inhibits cell proliferation of human thyroid carcinoma cell lines of both follicular and parafollicular origin. *J Endocrinol Invest* 25:362–368 (2002).
- Cerutti J, Trapasso F, Battaglia C, Zhang L, Martelli ML, Visconti R, Berlingieri MT, Fagin JA, Santoro M, Fusco A: Block of *c-myc* expression by antisense oligonucleotides inhibits proliferation of human thyroid carcinoma cell lines. *Clin Cancer Res* 2:119–126 (1996).
- Eichler EE, Hoffman SM, Adamson AA, Gordon LA, McCready P, Lamerdin JE, Mohrenweiser HW: Complex beta-satellite repeat structures and the expansion of the zinc finger gene cluster in 19p12. *Genome Res* 8:791–808 (1998).
- Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP: High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. *J Clin Invest* 91:179–184 (1993).
- Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6–13 (1983).
- Fredericks WJ, Ayyanathan K, Herlyn M, Friedman JR, Rauscher FJ III: An engineered PAX3-KRAB transcriptional repressor inhibits the malignant phenotype of alveolar rhabdomyosarcoma cells harboring the endogenous PAX3-FKHR oncogene. *Mol Cell Biol* 20:5019–5031 (2000).
- Friedman JR, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ III: KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev* 10:2067–2078 (1996).
- Kim SS, Chen YM, O'Leary E, Witzgall R, Vidal M, Bonventre JV: A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. *Proc natl Acad Sci, USA* 93:15299–15304 (1996).
- Margolin JF, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher FJ III: Kruppel-associated boxes are potent transcriptional repression domains. *Proc natl Acad Sci, USA* 91:4509–4513 (1994).
- Nawrath M, Pavlovic J, Moelling K: Inhibition of human hematopoietic tumor formation by targeting a repressor Myb-KRAB to DNA. *Cancer Gene Ther* 7:963–972 (2000).
- Pang XP, Hershman JM, Chung M, Pekary AE: Characterization of tumor necrosis factor-alpha receptors in human and rat thyroid cells and regulation of the receptors by thyrotropin. *Endocrinology* 125:1783–1788 (1989).
- Rippe V, Belge G, Meiboom M, Kazmierczak B, Fusco A, Bullerdiek J: A KRAB zinc finger protein gene is the potential target of 19q13 translocation in benign thyroid tumors. *Genes Chrom Cancer* 26:229–236 (1999).
- Shannon M, Ashworth LK, Mucenski ML, Lamerdin JE, Branscomb E, Stubbs L: Comparative analysis of a conserved zinc finger gene cluster on human chromosome 19q and mouse chromosome 7. *Genomics* 33:112–120 (1996).
- Vissing H, Meyer WK, Aagaard L, Tommerup N, Thiesen HJ: Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins. *FEBS Lett* 369:153–157 (1995).
- Witzgall R, O'Leary E, Leaf A, Onaldi D, Bonventre JV: The Kruppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. *Proc natl Acad Sci, USA* 91:4514–4518 (1994).
- Wu H, Zhang S, Qiu W, Zhang G, Xia Q, Xiao C, Huang X, Huang M, Agen P, Fan T, Yang J, Milunsky A: Isolation, characterization, and mapping of a novel human KRAB zinc finger protein encoding gene ZNF463. *Biochim biophys Acta* 1518:190–193 (2001).
- Zeki K, Nakano Y, Inokuchi N, Watanabe K, Morimoto I, Yamashita U, Eto S: Autocrine stimulation of interleukin-1 in the growth of human thyroid carcinoma cell line NIM 1. *J Clin Endocrinol Metab* 76:127–133 (1993).

# **XIII.**

## **Molecular characterization of the canine *HMGB1*.**

**Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM,  
Bullerdiek J, Nolte I.**

*Cytogenet Genome Res. 2003; 101(1): 33-8.*

Contribution to the work

- Planning and coordination of all done work
- Northern Blot Hybridisation
- Radioactive BAC screening
- *In silico* Analysis
- Composing of the publication

# Molecular characterization of the canine HMGB1

H. Murua Escobar,<sup>a,b</sup> B. Meyer,<sup>a</sup> A. Richter,<sup>a</sup> K. Becker,<sup>a</sup> A.M. Flohr,<sup>a</sup>  
J. Bullerdiek<sup>a</sup> and I. Nolte<sup>b</sup>

<sup>a</sup>Center for Human Genetics, University of Bremen, Bremen;

<sup>b</sup>Clinic for Small Animals, School of Veterinary Medicine Hannover, Hannover (Germany)

**Abstract.** Due to the close similarities of numerous canine diseases to their human counterparts, the dog could join the mouse as the species of choice to unravel the genetic background of complex diseases as e.g. cancer and metabolic diseases. Accordingly, the role of the dog as a model for therapeutic approaches is strongly increasing. However, prerequisite for such studies is the characterization of the corresponding canine genes. Recently, the human high mobility group protein B1 (HMGB1) has attracted considerable interest of oncologists because of what is called its “double life”. Besides its function as an architectural transcription factor HMGB1 can also be

secreted by certain cells and then acts as a ligand for the receptor for advanced glycation end products (RAGE). The binding of HMGB1 to RAGE can activate key cell signaling pathways, such as p38<sup>MAPK</sup>, JNK, and p42/p44<sup>MAPK</sup> emphasizing the important role of HMGB1 in inflammation and tumor metastasis. These results make HMGB1 a very interesting target for therapeutic studies done in model organisms like the dog. In this study we characterized the molecular structure of the canine HMGB1 gene on genomic and cDNA levels, its predicted protein, the gene locus and a basic expression pattern.

Copyright © 2003 S. Karger AG, Basel

As witnessed by a number of recent articles (Kuska, 1999; Kingman, 2000; Ostrander et al., 2000; Vail and MacEwen, 2000) a growing number of scientists predict that human genetics will be “going to the dogs” in this century (Kuska, 1999). Due to the emerging advantages of numerous canine diseases as a genetic model for human orthologs, the dog could join the mouse as the species of choice to unravel genetic mechanisms e.g. of cancer predisposition, development, and progression.

A very interesting group of genes in terms of oncology are the high mobility group (HMG) protein genes. High mobility group proteins named according to their electrophoretic mobility in the electric field are small chromatin-associated nonhistone proteins which can be subdivided into three families

because of their functional sequence motifs: the HMGA, HMGB, and HMGN protein families. Three human members of the HMGB protein family are presently known: the HMGB1, HMGB2, and HMGB3 proteins. The best analyzed member of this group, HMGB1 (synonymously known as HMG1 or amphoterin), can modify chromatin structure by bending DNA thus influencing the transcription of a number of target genes (for review see Muller et al., 2001). Recently, HMGB1 has attracted additional interest of oncologists because of what was called its “double life”. Besides its function as an architectural transcription factor, HMGB1 can also be secreted by certain cells, e.g. macrophages (Wang et al., 1999). As an extracellular protein HMGB1 is a ligand for the receptor for advanced glycation end products (RAGE) (Parkkinen and Rauvala 1991; Parkkinen et al., 1993; Hori et al., 1995) thus activating key cell signaling pathways, such as p38<sup>MAPK</sup>, JNK, and p42/p44<sup>MAPK</sup> and playing an important role in inflammation and tumor metastasis (Liotta and Clair, 2000; Taguchi et al., 2000).

The high mobility group protein HMGB1 has a number of features particularly related to the development and progres-

Received 9 August 2002; revision accepted 25 March 2003.

Request reprints from Dr. J. Bullerdiek, Center for Human Genetics  
University of Bremen, Leobener Strasse ZHG, D-28359 Bremen (Germany)  
telephone: +49-421-2184239; fax: +49-421-2184239  
e-mail: bullerdiek@uni-bremen.de

sion of gynaecological cancers. As an example HMGB1 has been shown to interact with and modify the binding affinity of several transcription factors, e.g. TATA-binding protein (TBP), Hox D9 protein, and steroid hormone receptors (Ge and Roeder, 1994; Zappavigna et al., 1996; Verrier et al., 1997; Boonyaratankornkit et al., 1998; Romine et al., 1998; Das and Scovell, 2001). Interestingly, HMGB1 gene expression can be induced by estrogens in breast cancer MCF-7 cells probably due to an upregulation of the gene so that HMGB1 itself can be considered an estrogen-responsive gene (Chau et al., 1998). Additionally, it has been shown that HMGB1 is able to bind to cisplatin-DNA adducts (Pil and Lippard, 1992) and sensitizes cancer cells to cisplatin by shielding its major DNA adducts from nucleotide excision repair (He et al., 2000). He et al. (2000) have shown that in estrogen receptor-positive human breast cancer cells estrogen can significantly increase the effect of cisplatin by causing an overexpression of HMGB1. This finding has led to the conclusion that estrogen treatment prior to cisplatin therapy may sensitize the cancer cells against that drug. Accordingly, a clinical trial for the treatment of gynaecological tumors with cisplatin has already been approved by the Food and Drug Administration (He et al., 2000).

However, the canine HMGB1 gene had not yet been characterized molecularly. The enlightenment of the molecular structure could permit new therapeutic approaches using the dog as model organism.

## Materials and methods

### *cDNA characterization*

Total RNA was isolated from 50 mg canine testis tissue using TRIZOL LS (Invitrogen) following the manufacturer's protocol. cDNA synthesis was performed using AP2 primer (AAGGATCCGTCGACATC(17)T), 5 µg total RNA and M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions. The PCRs for the molecular cloning of the cDNA were done using the following primer pairs: 5'-AGCTCCATAGAGACAG-CACC-3'/5'-TCTTCCTCCTCCTCATCC-3' and primer pair 5'-AGG-CCTCTTGGGTGCATT-3'/5'-AGTATCATCCAGGACTCAGAT-3'.

PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM T-easy vector system (Promega) and sequenced. The cDNA contig and the homology alignments were created with two sequences from the NCBI database (acc. nos. AF281043, U51677).

### *Protein sequence*

The canine HMGB1 protein sequence was derived from the ORF of the characterized cDNA sequence described previously. The protein homology alignments were created with sequences from the NCBI database (acc. nos. AF281043, U51677).

### *Northern blot*

Total RNAs were isolated from canine heart, lung, muscle, kidney, and spleen tissue using RNeasy system (QIAGEN). An additional sample of total RNA was isolated from canine heart tissue by TRIZOL LS acid guanidine isothiocyanate-chloroform method (Invitrogen) in order to figure out whether this isolation method leads to any difference in hybridization. Furthermore, mRNA was purified from canine spleen total RNA with OLIGOTEX (QIAGEN) and total RNA was prepared from human cultured fibroblasts by RNeasy system (QIAGEN). Spleen mRNA was placed on the blot in case that HMGB1 was not detectable in the total RNA samples.

For Northern blot hybridization 20 µg of total RNA of each sample (except muscle 10 µg and spleen mRNA 3.6 µg) were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred

onto Hybond-N+ positive nylon membrane (Amersham Pharmacia Biotech) by capillary blot.

A 292-bp cDNA fragment derived from the coding sequence (exon 2/exon 3) of the canine HMGB1 served as a molecular probe for hybridization. The probe was generated by PCR with the primer set 5'-AGGCTCTTGGGTGCATT-3'/5'-GGGCAAAGGAGATCCTAAGAAG-3' (Jiang et al., 1998) on the cloned cDNA described previously. Labeling was performed by random primed labeling (Roche Diagnostics) as described in the manufacturer's protocol with 50 µCi [ $\alpha^{32}$ P]dCTP (Amersham Pharmacia Biotech). Purification of the labeled probe was done using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at -20°C before use.

Using the PERFECTHYB PLUS hybridization solution (Sigma-Aldrich) prehybridization was carried out for 30 min and hybridization for 2.5 h at 68°C. The membrane was washed for 5 min at room temperature in 2× SSC, 0.1% SDS, and twice for 20 min at 68°C in 0.5× SSC, 0.1% SDS. Signals were visualized by using a STORM imager (Molecular Dynamics, Sunnyvale, USA).

### *BAC screening and FISH*

An HMGB1 canine genomic DNA probe was used for hybridization of canine RPCI 81 BAC/PAC filter (BACPAC RESOURCES, Children's Hospital Oakland Research Institute, Oakland, USA). The 531-bp probe was generated by PCR (Primer 5'-AGGCTCTTGGGTGCATT-3'/5'-AGAG-GCCTCCGTGAGTATCTTG-3') on genomic DNA of the canine pleomorphic adenoma cell line ZMTH3 (Center for Human Genetics, Bremen, Germany). The obtained PCR product was separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM T-easy vector system (Promega) and sequenced for verification. The probe labeling was performed by random primed labeling (Roche Diagnostics) as described in the manufacturer's protocol with 50 µCi [ $\alpha^{32}$ P]dCTP (Amersham Pharmacia Biotech). Purification of the labeled probe was done using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at -20°C before use.

The filters were placed in a minimum volume of Church Buffer (0.15 mM BSA, 1 mM EDTA, 0.5 M NaHPO<sub>4</sub>, 7% SDS) and transferred into hybridization bottles. The filters were prehybridized at 65°C for 1 h in 25 ml Church Buffer. Hybridization was performed at 65°C overnight (16–18 h) in the same solution. All further steps were performed according to manufacturer's protocol. Signals were visualized using a STORM imager (Molecular Dynamics, Sunnyvale, USA).

Metaphase preparations were obtained from blood samples of two different dogs. The samples were stimulated with PHA and cultured for 96 h at 37°C. After 1.5 h colcemide (0.1 µg/ml) incubation, the lymphocytes were harvested according to routine procedures. Prior to FISH, chromosomes were stained using the GTG-banding method. After taking photographs of the metaphases, the slides were destained in 70% ethanol for 15 min and air dried.

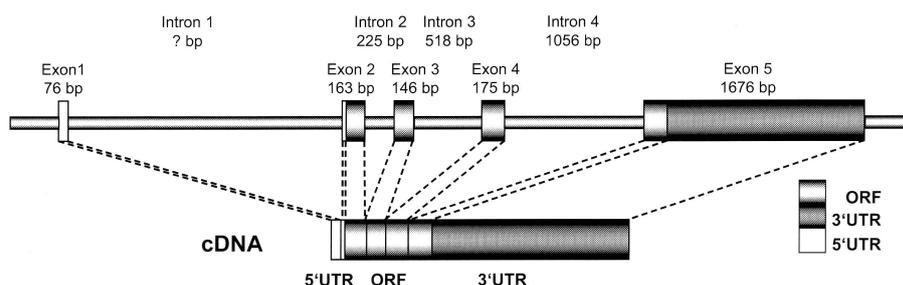
FISH was performed using the protocol of Reimann et al. (1996) with some modifications. BAC DNA was digoxigenin labeled (Dig-Nick-Translation-Kit, Roche Diagnostics). The hybridization mixture contained 125–175 ng probe, 43.2 µg salmon sperm DNA, 1,000–1,200 ng sonicated dog DNA, 2× SSC, 2× SSPE, 50% formamide and 10% dextran sulfate. The chromosomes were stained with propidium iodide and G-bands were identified according to Reimann et al. (1996).

### *Genomic characterization*

For genomic characterization of the canine HMGB1 gene the introns were amplified by PCR on the screened BAC 24-A16 (BACPAC RESOURCES, Children's Hospital Oakland Research Institute, Oakland, USA) and on genomic DNA of the canine pleomorphic adenoma cell line ZMTH3 (Center for Human Genetics, Bremen, Germany). For intron 2 a 517-bp fragment was generated with primer pair 5'-AGGCTCTTGGGTGCATT-3'/5'-GGGCAAAGGAGATCCTAAGAAG-3', a 579-bp intron 3 fragment was generated by the primer pair 5'-GATCCCAATGCACCCAAGAG-3'/5'-GGGGATACTCAGAACAAAACA-3' and the fourth exon 1,224-bp fragment was generated with pair 5'-GAAGGCTGCTAAGCTGAAGGA-3'/5'-TCTTCCTCCTCCTCATCC-3'. The obtained PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM T-Easy vector system (Promega) and sequenced for verification. The final genomic canine HMGB1 contig and the homology alignments were

## Canine HMGB1

### putative genomic structure



**Fig. 1.** Structure of the genomic elements and the cDNA of the canine HMGB1.

created with Lasergene software (DNASar, Madison, USA) with the generated sequences from the cloned cDNA described previously and various sequences from the NCBI database (acc. nos. AF 281043, U 51677, NM\_002128).

### Results

Herein, we describe the genomic and the cDNA structure, the predicted protein sequence, a basic expression pattern, and the chromosomal locus of the canine HMGB1 gene.

#### cDNA sequence

The canine cDNA sequence consists of 2,236 bp spanning five exons. The exon structure, the UTRs, and the ORF were defined and their homologies to their human counterpart analyzed (Fig. 1, Table 1). The cDNA sequence was submitted to the NCBI database acc. no. AY135519.

#### Protein sequence

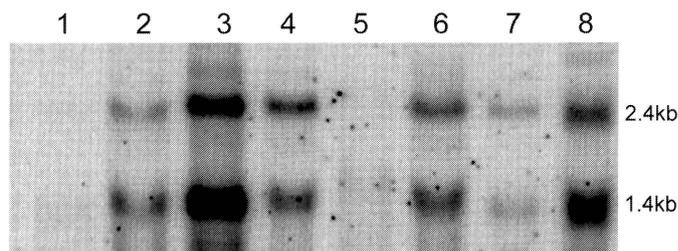
The canine HMGB1 protein sequence was deduced from the composite cDNA sequence. The protein is a 215-amino-acid (AA) molecule with a calculated weight of 24,892.67 Daltons (Fig. 3, Table 2). The sequence was submitted to the NCBI database acc. no. AY135519. Homology comparison to the human counterpart showed 100% homology of the molecules including the two "HMG boxes" and the acidic carboxy-terminal domain, while mouse and bovine molecules showed differences in the acidic tail.

#### Genomic structure

The genomic structure of the canine HMGB1 gene consists of the previously described five exons and four introns. Due to the fact that intron 1 could not be cloned a contig spanning exon 2 to exon 5 consisting of 3,959 bp was created. The exon/intron structure, size and the homologies to their human counterparts were analyzed and defined (Fig. 1, Table 1). The genomic sequences were submitted to the NCBI database (acc. nos. AY135520, AY135521).

#### Northern blot

To elucidate a basic expression pattern, a canine Northern blot was performed using RNA and mRNA from canine spleen,



**Fig. 2.** Northern blot showing 1.4-kb and 2.4-kb HMGB1 transcripts. Lanes: canine: (1) kidney, (2, 3) spleen, (4, 5) heart, (6) lung, (7) muscle; human: (8) fibroblasts.

**Table 1.** Detailed analysis of the canine HMGB1 cDNA and genomic elements: Homology comparison of the genomic and cDNA elements of the canine HMGB1 to its human counterpart (Characterisation of the UTRs, the ORF and the exon/intron sizes)

Element of canine HMGB1	Size in bp	Homology to human counterpart in %
<i>cDNA elements</i>		
Total cDNA	2236	90.8
5' UTR	89	95.6
cds	648	95.4
3' UTR	1499	88.6
<i>Genomic elements</i>		
Total Exon 2-5	3959	73.3
Exon 1	76	98.7
Intron 1	-	-
Exon 2	163	95.2
Intron 2	225	20.3
Exon 3	146	95.2
Intron 3	518	59.0
Exon 4	175	93.7
Intron 4	1056	61.8
Exon 5	1676	89.4

heart, lung, muscle, and kidney tissue samples and hybridized with a <sup>32</sup>P-labeled canine HMGB1 cDNA probe. Except for the kidney tissue that showed no detectable signal, all samples revealed two transcripts similar to the signals obtained in human fibroblasts (Fig. 2) of about 1.4 and 2.4 kb. One of two

	10	20	30	40	
1	M G K G D P K K P R G K M S S Y A F F V Q T C R E E H K K K H P D A S V N F S E				Canine
1	M G K G D P K K P R G K M S S Y A F F V Q T C R E E H K K K H P D A S V N F S E				Human
1	M G K G D P K K P R G K M S S Y A F F V Q T C R E E H K K K H P D A S V N F S E				Bovine
1	M G K G D P K K P R G K M S S Y A F F V Q T C R E E H K K K H P D A S V N F S E				Mouse
	50	60	70	80	
41	F S K K C S E R W K T M S A K E K G K F E D M A K A D K A R Y E R E M K T Y I P				Canine
41	F S K K C S E R W K T M S A K E K G K F E D M A K A D K A R Y E R E M K T Y I P				Human
41	F S K K C S E R W K T M S A K E K G K F E D M A K A D K A R Y E R E M K T Y I P				Bovine
41	F S K K C S E R W K T M S A K E K G K F E D M A K A D K A R Y E R E M K T Y I P				Mouse
	90	100	110	120	
81	P K G E T K K K F K D P N A P K R P P S A F F L F C S E Y R P K I K G E H P G L				Canine
81	P K G E T K K K F K D P N A P K R P P S A F F L F C S E Y R P K I K G E H P G L				Human
81	P K G E T K K K F K D P N A P K R P P S A F F L F C S E Y R P K I K G E H P G L				Bovine
81	P K G E T K K K F K D P N A P K R P P S A F F L F C S E Y R P K I K G E H P G L				Mouse
	130	140	150	160	
121	S I G D V A K K L G E M W N N T A A D D K Q P Y E K K A A K L K E K Y E K D I A				Canine
121	S I G D V A K K L G E M W N N T A A D D K Q P Y E K K A A K L K E K Y E K D I A				Human
121	S I G D V A K K L G E M W N N T A A D D K Q P Y E K K A A K L K E K Y E K D I A				Bovine
121	S I G D V A K K L G E M W N N T A A D D K Q P Y E K K A A K L K E K Y E K D I A				Mouse
	170	180	190	200	
161	A Y R A K G K P D A A K K G V V K A E K S K K K K E E E E E				Canine
161	A Y R A K G K P D A A K K G V V K A E K S K K K K E E E E E				Human
161	A Y R A K G K P D A A K K G V V K A E K S K K K K E E E E E				Bovine
161	A Y R A K G K P D A A K K G V V K A E K S K K K K E E E E E				Mouse
	210				
201	E D E E D E D E E E D D D D E				Canine
201	E D E E D E D E E E D D D D E				Human
201	E D E E D E E E E E D D D D E				Bovine
201	E E E E D E D E E E D D D D E				Mouse

Fig. 3. Comparison of canine, human, mouse, and bovine HMGB1 proteins.

Table 2. Detailed in silico analysis of the canine HMGB1 protein

Amino Acids (AA)	Molecular Weight in Da	Isoelectric Point	Charge at pH 7.0
215	24892.67	5.591	-4.629
Strongly Basic (+) AA	Strongly Acidic (-) AA	Hydrophobic AA	Polar AA
51	56	43	32

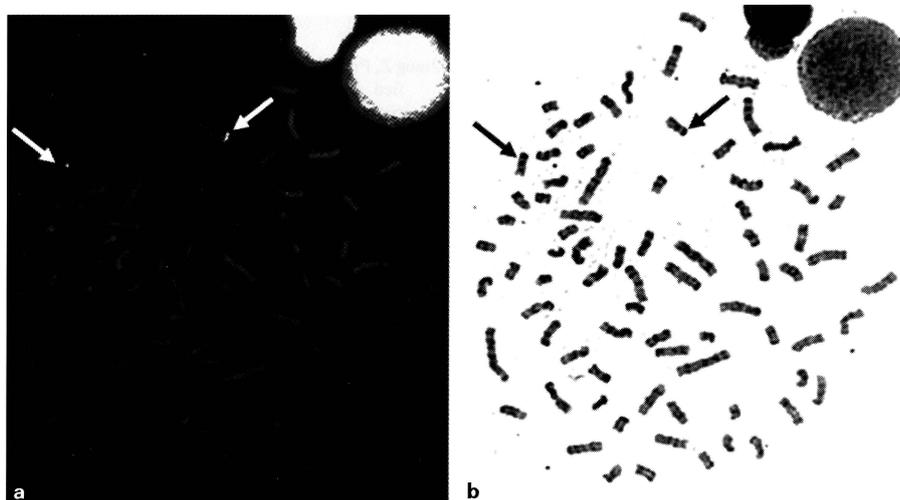
canine heart samples showed weak signals probably due to the Trizol sample quantification difficulties. Human HMGB1 transcripts of this size had been detected in multiple normal tissues and several breast cancer samples.

#### BAC screening and FISH

A canine HMGB1 genomic DNA probe was generated and used for screening a canine BAC for localization of the canine HMGB1 gene locus by FISH.

The screened BAC 24-A16 was first verified according to the manufacturer's protocol which requires two hybridization signals to appear in a specific orientation on the filter spotting panel. Second verification was done by PCR using primer pair UP: 5'-GAAGGCTGCTAAGCTGAAGGA-3'/LO: 5'-TCTTCCTCCTCCTCATCC-3' spanning intron 4. For final verification the obtained PCR product was separated on agarose gel, recovered, cloned and sequenced as described previously.

**Fig. 4.** An example of a metaphase spread after FISH with signals on both chromosomes 25 (a) and the same metaphase after GTG-banding (b).



The verified BAC was used for FISH experiments. Sixteen well spread metaphases were examined for analysis. Signals were detected on both chromatides of both chromosomes. The obtained signals mapped the canine gene to CFA25 (Fig. 4).

## Discussion

The human HMGB1 is considered to have a “double life” with impact on several diseases including tumorigenesis (Taguchi et al., 2000; Flohr et al., 2001). First, by acting as an architectural transcription factor, HMGB1 influences the formation of transcription factor complexes of several target genes. Second, HMGB1 can be released from some cells allowing its binding to the cell surface receptor RAGE. Blockade of RAGE-HMGB1 interaction in rat C6 glioma cells significantly inhibits their growth, motility, and local invasion as well as metastasis (Liotta and Clair, 2000; Taguchi et al., 2000).

The aim of the present study was to characterize the cDNA, protein, genomic structure, gene locus, and a basic expression pattern of the canine HMGB1 gene. Knowledge of its structure would be the precondition to evaluate the canine gene product as a potential target for therapeutic approaches using the dog as model system.

The complete canine HMGB1 cDNA consists of 2,236 bp encoded by five exons similar to the human transcript (acc. no. AY135519). The homology of the complete molecule to its human counterpart is 90.8% whereas the ORF is 95.4%. The derived canine protein consists of 215 AA with a molecular weight of 24,892.67 Da. Comparison to the human protein showed 100% homology of the canine counterpart. Differences between human HMGB1 and the mouse protein were described as two or three AA changes in the acidic carboxy-terminal domain (Paonessa et al., 1987; Ferrari et al., 1994). The bovine molecule (acc. no. P10103) shows one AA change compared to its human and canine counterpart. Due to the previously described properties of the canine HMGB1 and the identical structure to the human molecule, therapeutic approaches in dogs likely can be performed with identical compounds.

Northern blot analysis was performed to define a basic expression pattern in canine heart, lung, muscle, kidney, and spleen tissue. Except for the kidney tissue that showed no detectable signal, all samples revealed two transcripts similar to the signals obtained in human fibroblasts of about 1.4 and 2.4 kb. One of two canine heart samples showed weak signals probably due to the Trizol sample quantification difficulties. Human HMGB1 transcripts of this size had been detected in multiple normal tissues (Rogalla et al., 1998) and several breast cancer samples (Flohr et al., 2001).

At the genomic level the canine HMGB1 gene exon/intron structure is similar to the human ortholog consisting of five exons and four introns. While the homologies of the exons, ranging between 89.4 and 98.7%, are extremely high, the homologies of the amplified introns 2–4 vary between 20.3 and 59.0%. Approaches to amplify the canine intron 1 resulted in unspecific PCR products which were probably due to the putative existence of various CpG islands found in human intron 1 (Borrmann et al., 2001).

Mapping of the canine HMGB1 gene resulted in assignment to CFA25. The G-bands were identified according to Reimann et al. (1996).

Yang et al. (1999) found no conservation of synteny between the human chromosome 13 where the human HMGB1 is located and the canine chromosome 25 where we mapped canine HMGB1.

Nevertheless the molecular characterization of the canine HMGB1 gene and protein showed clearly that man and dog share an identical HMGB1 protein. Considering the similarities of genesis and development of diseases in both species molecular targeting of HMGB1 in dogs can be of significant importance for therapeutic approaches in humans as well.

## References

- Boonyaratankornkit V, Melvin V, Prendergast P, Altmann M, Ronfani L, Bianchi ME, Taraseviciene L, Nordeen SK, Allegretto EA, Edwards DP: High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. *Mol cell Biol* 18:4471–4487 (1998).
- Borrmann L, Kim I, Schultheiss D, Rogalla P, Bullerdiek J: Regulation of the expression of HMG1, a co-activator of the estrogen receptor. *Anticancer Res* 21:301–305 (2001).
- Chau KY, Lam HY, Lee KL: Estrogen treatment induces elevated expression of HMG1 in MCF-7 cells. *Exp Cell Res* 241:269–272 (1998).
- Das D, Scovell WM: The binding interaction of HMG1 with the TATA-binding protein/TATA complex. *J Biol Chem* 276:32597–32605 (2001).
- Ferrari S, Ronfani L, Calogero S, Bianchi ME: The mouse gene coding for high mobility group 1 protein (HMG1). *J Biol Chem* 269:28803–28808 (1994).
- Flohr AM, Rogalla P, Meiboom M, Borrmann L, Krohn M, Thode-Halle B, Bullerdiek J: Variation of HMGB1 expression in breast cancer. *Anticancer Res* 21:3881–3885 (2001).
- Ge H, Roeder RG: The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein. *J Biol Chem* 269:17136–17140 (1994).
- He Q, Liang CH, Lippard SJ: Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. *Proc Natl Acad Sci, USA* 97:5768–5772 (2000).
- Hori O, Brett J, Slattey T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, Morser J, Stern D, Schmidt AM: The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphotericin. Mediation of neurite outgrowth and co-expression of RAGE and amphotericin in the developing nervous system. *J Biol Chem* 270:25752–25761 (1995).
- Jiang Z, Priat C, Galibert F: Traced orthologous amplified sequence tags (TOASTs) and mammalian comparative maps. *Mammal Genome* 9:577–787 (1998).
- Kingman S: Painting a brighter future for dogs and humans. *Drug Discovery Today* 5:127–128 (2000).
- Kuska B: Sit, DNA, sit: cancer genetics going to the dogs. *J Natl Cancer Inst* 91:204–209 (1999).
- Liotta LA, Clair T: Cancer. Checkpoint for invasion. *Nature* 405:287–288 (2000).
- Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME: New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J* 20:4337–4340 (2001).
- Ostrander EA, Galibert F, Patterson DF: Canine genetics comes of age. *Trends Genet* 16:117–124 (2000).
- Paonessa G, Frank R, Cortese R: Nucleotide sequence of rat liver HMG1 cDNA. *Nucl Acids Res* 15:9077 (1987).
- Parkkinen J, Rauvala H: Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphotericin. Enhancement of t-PA-catalyzed plasminogen activation by amphotericin. *J Biol Chem* 266:16730–16735 (1991).
- Parkkinen J, Raulo E, Merenmies J, Nolo R, Kajander EO, Baumann M, Rauvala H: Amphotericin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. *J Biol Chem* 268:19726–19738 (1993).
- Pil PM, Lippard SJ: Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science* 256:234–237 (1992).
- Reimann N, Bartnizke S, Bullerdiek J, Schmitz U, Rogalla P, Nolte I, Ronne M: An extended nomenclature of the canine karyotype. *Cytogenet Cell Genet* 73:140–144 (1996).
- Rogalla P, Borda Z, Meyer-Bolte K, Tran KH, Hauke S, Nimzyk R, Bullerdiek J: Mapping and molecular characterization of five HMG1-related DNA sequences. *Cytogenet Cell Genet* 83:124–129 (1998).
- Rogalla P, Kazmierczak B, Flohr AM, Hauke S, Bullerdiek J: Back to the roots of a new exon – the molecular archaeology of a SP100 splice variant. *Genomics* 63:117–122 (2000).
- Romine LE, Wood JR, Lamia LA, Prendergast P, Edwards DP, Nardulli AM: The high mobility group protein 1 enhances binding of the estrogen receptor DNA binding domain to the estrogen response element. *Mol Endocrinol* 12:664–674 (1998).
- Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM: Blockade of RAGE-amphotericin signalling suppresses tumor growth and metastases. *Nature* 405:354–360 (2000).
- Vail DM, MacEwen EG: Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest* 18:781–792 (2000).
- Verrier CS, Roodi N, Yee CJ, Bailey LR, Jensen RA, Bustin M, Parl FF: High-mobility group (HMG) protein HMG-1 and TATA-binding protein-associated factor TAF(II)30 affect estrogen receptor-mediated transcriptional activation. *Mol Endocrinol* 11:1009–1019 (1997).
- Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ: HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 285:248–251 (1999).
- Yang F, O'Brien PCM, Milne S, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D, Ferguson-Smith MA: A Complete Comparative Chromosome Map for the Dog, Red Fox, and Human and its Integration with Canine Genetic Maps. *Genomics* 62:189–202 (1999).
- Zappavigna V, Falciola L, Helmer-Citterich M, Mavilio F, Bianchi ME: HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J* 15:4981–4991 (1996).

# XIV.

## **Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas.**

**Rippe V, Drieschner N, Meiboom M, Murua Escobar H, Bonk U,  
Belge G, Bullerdiek J.**

*Oncogene. 2003; 22(38): 6111-4.*

Contribution to the work

- Radioactive probe labelling
- Northern Blot Hybridisation
- Blot imaging

# Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas

Volkhard Rippe<sup>1</sup>, Norbert Drieschner<sup>1</sup>, Maren Meiboom<sup>1</sup>, Hugo Murua Escobar<sup>1</sup>, Ulrich Bonk<sup>2</sup>, Gazanfer Belge<sup>1</sup> and Jörn Bullerdiek<sup>\*1</sup>

<sup>1</sup>Center for Human Genetics, University of Bremen, Leobener Str. ZHG, D-28359 Bremen, Germany; <sup>2</sup>Institute of Pathology, Central Hospital Bremen-Nord, Bremen, Germany

Thyroid adenomas belong to the cytogenetically best investigated human epithelial tumors. Cytogenetic studies of about 450 benign lesions allow one to distinguish between different cytogenetic subgroups. Two chromosomal regions, that is, 19q13 and 2p21, are frequently rearranged in these tumors. Although 2p21 aberrations only account for about 10% of the benign thyroid tumors with clonal cytogenetic deviations, 2p21 rearrangements belong to the most common cytogenetic rearrangements in epithelial tumors due to the high frequency of these benign lesions. The 2p21 breakpoint region recently has been delineated to a region of 450 kbp, but the gene affected by the cytogenetic rearrangements still has escaped detection. Positional cloning and 3' RACE-PCR allowed us to clone that gene which we will refer to as thyroid adenoma associated (*THADA*) gene. In cells from two thyroid adenomas characterized by translocations t(2;20;3)(p21;q11.2;p25) and t(2;7)(p21;p15), respectively, we performed 3'-RACE-PCRs and found two fusions of *THADA* with a sequence derived from chromosome band 3p25 or with a sequence derived from chromosome band 7p15. The *THADA* gene spans roughly 365 kbp and, based on preliminary results, encodes a death receptor-interacting protein.

Oncogene (2003) 22, 6111–6114. doi:10.1038/sj.onc.1206867

**Keywords:** 2p21 aberrations; thyroid adenomas; THADA; fusion gene

## Introduction

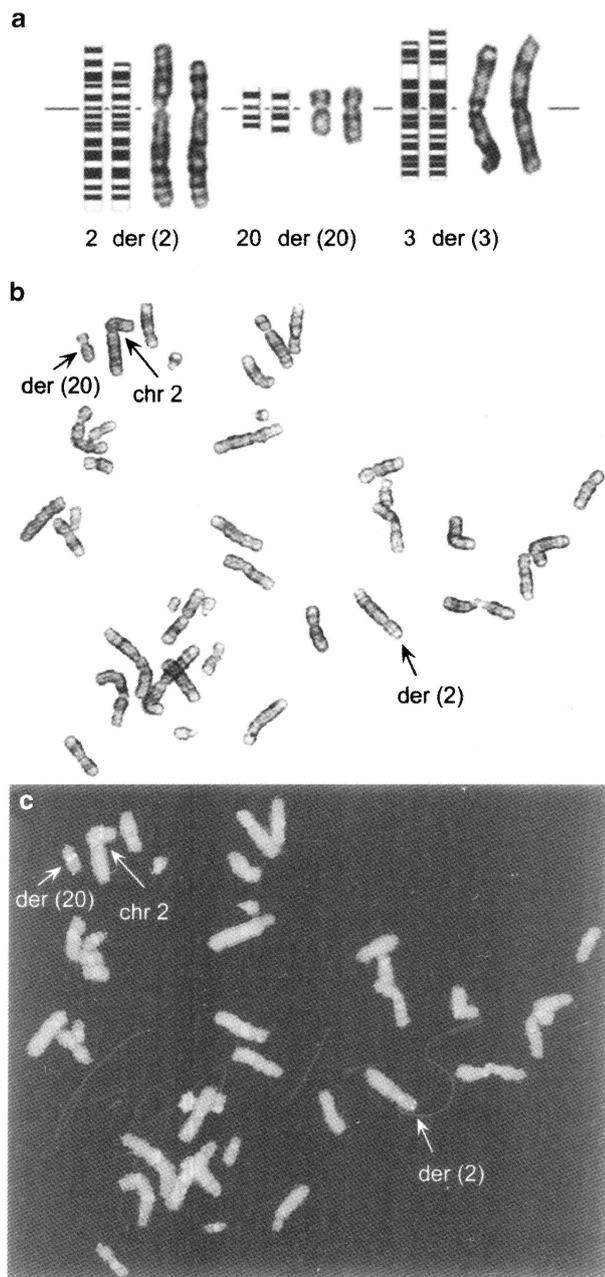
Adenomas of the thyroid are rare examples of epithelial tumors frequently displaying specific chromosomal translocations. There are two chromosomal bands most frequently rearranged by these translocations, that is, 19q13 (Bartnitzke *et al.*, 1989; Bondeson *et al.*, 1989; Dal Cin *et al.*, 1992; Belge *et al.*, 1998) and 2p21 (Teyssier *et al.*, 1990; Belge *et al.*, 1998; Bol *et al.*, 1999). Both types of translocations apparently do not co-occur in thyroid tumors, thus indicating two independent cytogenetic subgroups. As for the translocation partners

there is also no apparent overlap and in both groups other chromosomal regions involved in the translocations remarkably vary. Nevertheless, there are preferred translocation partners that are more often involved than others, as for example chromosomal band 5q13 in cases with 19q13 translocations and band 7p15 in cases with 2p21 translocations. Whereas the breakpoint region of chromosome 19 has been characterized molecularly in detail and candidate genes have been found (Rippe *et al.*, 1999; Belge *et al.*, 2001), for the 2p21 region only a rough mapping of the breakpoint has been obtained with no candidate genes identified so far (Bol *et al.*, 2001). Herein, we describe the identification of the target gene of the 2p21 aberrations which we have tentatively referred to as thyroid adenoma-associated gene (*THADA*). The identification of genes affected by these aberrations and the elucidation of their mechanisms of action certainly will lead to better insights into the pathogenesis of these frequent diseases.

## Results

In the thyroid adenoma cell line S325/TSV40 characterized by a translocation t(2;20;3)(p21;q11.2;p25), we found a single chromosome 2 BAC clone, 1069E24, that hybridized to both the der(2) and the der(20) chromosome and thus contained the altered 2p21 gene locus (Bol *et al.*, 2001) (Figure 1). Database search based on clone 1069E24 and the flanking BACs 339H12, 183F15, and 204D19 identified numerous anonymous human ESTs (BLAST analysis, version 2.2.6; Altschul *et al.*, 1997). Alignment and RT-PCR analyses point to a novel gene that we referred to as *THADA* (Figure 2). *THADA* cDNA contains 38 exons with at least one alternative splice variant (Figure 3). As for its possible function, *THADA* corresponds to the *death receptor-interacting protein* mRNA encoding a protein that in two-hybrid experiments was found to be a ligand of death receptor DR5 (Puduvalli VK and Ridgway L, GenBank accession reference note). The hypothetical 1954 amino acids (predicted molecular weight: 220 kDa) have no apparent homology to other human proteins as retrieved from public databases. Nevertheless, there are homologies to undefined ESTs in other organisms, as for example the mouse.

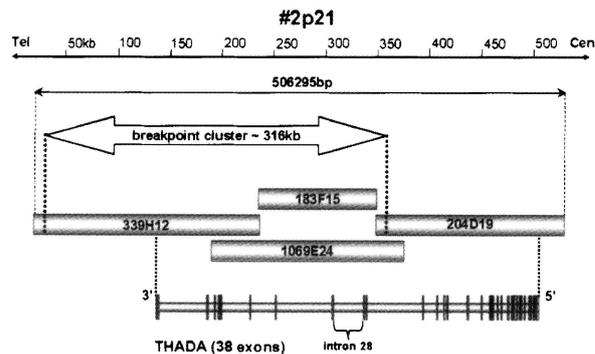
\*Correspondence: J Bullerdiek; E-mail: bullerdiek@uni-bremen.de  
Received 24 January 2003; revised 11 June 2003; accepted 13 June 2003



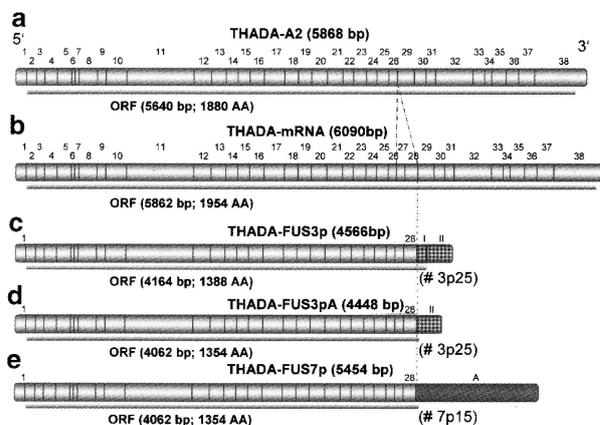
**Figure 1** Molecular cytogenetic investigations of the thyroid adenoma cell line S325/TSV40 with a translocation t(2;20;3)(p21;q11.2;p25). (a) Partial G-banded karyotype and schematic representation of the chromosomal aberrations showing normal (left) and derivative (right) chromosomes 2, 20, and 3, respectively. (b) Part of a metaphase of the cell line S325/TSV40 with t(2;20;3)(p21;q11.2;p25) after G-banding; the chromosomes 2, der(2), and der(20) are marked (arrows). (c) The same metaphase after FISH with BAC 1069E24; the hybridization signals are located on chromosomes 2, der(2), and der(20) (arrows)

The gene is widely expressed as revealed by *in silico* analyses using serial analysis of gene expression (SAGE) and EST-library database.

EST data show a high expression in adult normal placenta and SAGE data point to a strong expression



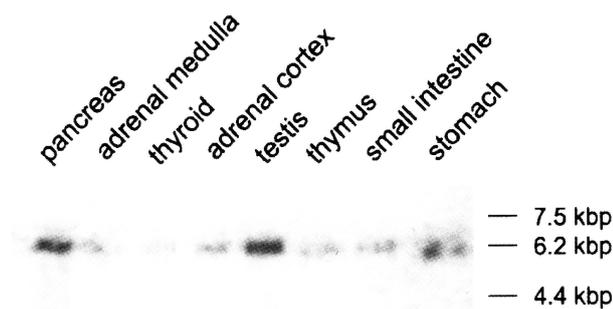
**Figure 2** Genomic organization of the breakpoint cluster region in thyroid lesions with aberrations of chromosomal band 2p21 and *THADA*. The BAC clones spanning the breakpoint region are represented as gray bars. The genomic structure of *THADA* is shown below. The breakpoints of the cell lines S325/TSV40 and S533/TSV40 are located in the intron 28 of *THADA*



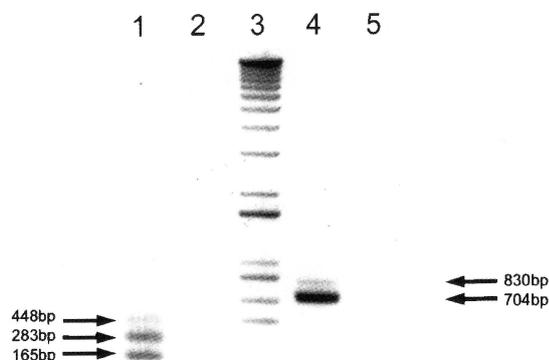
**Figure 3** mRNA organization of *THADA*, *THADA-FUS3p*, and *THADA-FUS7p*. (a) Splice variant of *THADA* (*THADA-A2*) lacking exons 27 and 28. (b) mRNA structure of *THADA* consisting of 38 exons. (c) mRNA structure of *THADA-FUS3p*. *THADA* stops after exon 28 followed by two 'exons' of the fusion part from chromosome region 3p25 (exons I and II). 3p25 fusion sequences are marked by checked pattern. (d) mRNA structure of *THADA-FUS3pA*. After exon 28, only 'exon' II of *FUS3p* is fused to *THADA*. (e) mRNA structure of *THADA-FUS7p*. The fusion part from chromosome region 7p15 (exon A) is fused to *THADA* downstream of exon 28 (marked by dot pattern). Under each mRNA, the potential ORF is shown as thin gray bar

in breast cancer cell line MCF7 and normal breast epithelium. A multiple Northern blot was probed with an 820 bp fragment from the ORF allowing us to detect a 6.2 kbp signal in all tissues investigated (Figure 4).

To identify possible *THADA* fusion partners, we used exon 18 and 28 oligonucleotide primers to perform 3'-RACE-PCR in cell lines S325/TSV40 and S533/TSV40. Amplification products were cloned and sequenced leading to the identification of clones in which the sequence diverged from *THADA* after exon 28. A database search revealed that the sequence fused to *THADA* in S325/TSV40 (*FUS3p*) maps to



**Figure 4** Expression studies of *THADA*. Hybridization of part of the cDNA of *THADA* (exons 25–32) on a commercially available poly(A)<sup>+</sup> RNA Northern blot revealed a transcript of about 6.2 kbp of varying intensity in all tissues. A strong expression of *THADA* is seen in pancreas and testis, thyroid tissue shows a weak expression of *THADA*



**Figure 5** RT-PCR detection of *THADA* fusion genes in the cell lines S325/TSV40 and S533/TSV40. Primers are located within exon 28 of *THADA* and the novel sequences derived from the corresponding translocation partners of chromosome 2. In lane 1, three amplification products of the cell line S325T/SV40 representing the three splice variants (165, 283 and 448 bp) of *THADA-FUS3p* were seen. In S533T/SV40, the fusion transcripts *THADA-FUS7p* were detected as 704 and 830 bp PCR products, as shown in lane 4. Both RT-PCRs confirmed the existence of *THADA-FUS3p* and *THADA-FUS7p*. In lanes 2 and 5, RT-PCR analyses with the same primers used on the cell lines were performed on normal thyroid with no detectable products. DNA molecular weight marker 1 kb-plus ladder (Invitrogen, Karlsruhe, Germany) is seen in lane 3

chromosomal band 3p25, BAC 167M22 and in S533/TSV40 (*FUS7p*) to chromosomal band 7p15, BAC 379L16 (Figure 3). By database search we have not been able to correlate these sequences to any described gene structures.

In order to confirm the fusions in both cell lines, RT-PCR analyses were performed. Amplification products corresponding to those found by RACE-PCR were obtained for both cell lines, thus confirming the *THADA-FUS3p* and *THADA-FUS7p* fusions (Figure 5). In both cell lines, *THADA* stops after exon 28 followed by the fused sequences from either of the translocation partners.

## Discussion

Chromosome 2p21 aberrations characterize the second most frequent structural chromosomal rearrangements in benign adenomas of the thyroid (Belge *et al.*, 1998; Bol *et al.*, 1999). From the results presented herein, there is little doubt that *THADA* is indeed the target gene of those translocations. In the translocations observed in both cell lines, the identical part of *THADA* becomes fused to ectopic sequences both mapping to the break-points of the translocation partners. Since no similarity of the ectopic sequences with known genes became apparent, it seems tempting to speculate that the truncation of the gene and the protein rather than its fusion to particular coding sequences is the critical event. As for the function of the *THADA* protein, there is so far only one unpublished report based on two-hybrid experiments indicating the involvement of *THADA* in death receptor pathway (Puduvalli VK and Ridgway L, GenBank accession reference note). We hypothesize that the truncation of the gene, and the loss of the 3' part of *THADA* leads to an altered apoptosis induction. This may cause an increased proliferation in benign thyroid tumors with chromosome 2p21 alterations. Previous studies revealed that truncation of genes could be an important event in human tumor development. For example, truncation of the 3' part of the high-mobility group gene *HMG2* could be relevant for the etiology of human sarcomas (Berner *et al.*, 1997) or causes malignant transformation of NIH3T3 cells (Fedele *et al.*, 1998). Apparently, further elucidation of the function of the *THADA* protein will be helpful to get insights into the molecular pathogenesis of benign adenomas of the thyroid and possibly also into benign proliferation in general.

## Materials and methods

### FISH analysis

For chromosome analyses and FISH studies, materials of the cell lines S325/TSV40 and S533/TSV40, derived from a thyroid adenoma, were used. The cell lines were obtained by transfection with a construct containing the SV40 large T antigen, as reported previously (Belge *et al.*, 1992). Cell culture of the cell lines and chromosome analyses were performed as previously described for pleomorphic adenomas of the parotid gland (Bullerdiek *et al.*, 1987). The BAC clones were obtained from the RP-11-Library (RZPD, Heidelberg, Germany). DNA of BAC clones was isolated using the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany). FISH analyses were performed after GTG banding of the same metaphase spreads. Treatment of metaphases and subsequent FISH experiments were performed using the protocol of Kievits *et al.* (1990). For FISH studies, BAC DNA was labeled either with dig-11-dUTP or biotin-16-dUTP by nick translation (Roche Diagnostics, Mannheim, Germany). For two-color FISH experiments, the labeled BAC DNA was pooled (concentration: 4 ng/ $\mu$ l). For one-color FISH, 4 ng/ $\mu$ l of each BAC clone was used. Detection was performed with anti-dig-fluorescein fab-fragments (Roche Diagnostics, Mannheim, Germany) and Cy3-conjugated streptavidin (Jackson Immuno Research, West

Grove, PA, USA). Chromosomes were counterstained with DAPI (0.025 µg/ml). For each cell line, at least 10 metaphases were examined. Slides were analysed on a Zeiss Axiophot fluorescence microscope using an FITC, Cy3, and DAPI filter set. Metaphases were recorded with the Power Gene Karyotyping System (Applied Imaging, Newcastle, UK).

#### PCR analyses

Total RNA was isolated from cells of S325/TSV40 and S533/TSV40 using TRIzol reagent (Invitrogen, Karlsruhe, Germany). Total RNA (5 µg) was reverse transcribed into first-strand cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). For 3'-RACE-PCR, a gradient thermocycler (Biometra, Göttingen, Germany) was used. Amplifications were carried out in a final volume of 50 µl containing 1.5 µl template cDNA, 1 × AdvanTaqPlus buffer, 0.2 µM of each primer, 200 µM dNTP mix, and 1 µl 50 × AdvanTaq cDNA Polymerase Mix. Primer sequences used for 3'-RACE were: 5'-GCTTCAGGCAGCAGCAGCATTTCCTCA-3' (exon 18) and 5'-AGACTCTACGCTTCCCCGATGGATGGT-3' (exon 28). The PCR reaction was performed according to the Advantage cDNA PCR Kit manual (Clontech, Palo Alto, CA, USA). After an initial denaturation at 94°C for 5 min, 35 cycles were performed. Each cycle consisted of denaturation (94°C) for 30 s and a combined annealing/extension step for 6 min at 68°C. 3'-RACE was completed with a final extension for 6 min at 68°C. RACE products were separated on a 1.2% agarose gel and transferred onto nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Little Chalfont, England) in 20 × SSC followed by UV crosslinking. Probe DNA (201 bp) was labeled with dig-11-dUTPs in a PCR reaction on a cDNA clone containing a *THADA*-sequence using primers 5'-GCTTCAGGCAGCAGCAGCATTTCCTCA-3' and 5'-ATTGGGATGAGGCCTTCAGGGGATGA-3'. Labeling, hybridization, and detection were performed according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). RT-PCR detection of the fusion genes was performed with primers 5'-AGACTCTACGCTTCCCCGATGGATGGT-3' 5'-TCCAGGGAAATTCAGTCTTG GGAGCCA-3' (*THADA-FUS3p*) and 5'-AGACTCTACG

#### References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. (1997). *Nucleic Acids Res.*, **25**, 3389–3402.
- Bartnitzke S, Herrmann ME, Lobeck H, Zuschneid W, Neuhaus P and Bullerdiek J. (1989). *Cancer Genet. Cytogenet.*, **39**, 65–68.
- Belge G, Kazmierczak B, Meyer-Bolte K, Bartnitzke S and Bullerdiek J. (1992). *Cell. Biol. Int. Rep.*, **16**, 339–347.
- Belge G, Rippe V, Meiboom M, Drieschner N, Garcia E and Bullerdiek J. (2001). *Cytogenet. Cell Genet.*, **93**, 48–51.
- Belge G, Roque L, Soares J, Bruckmann S, Thode B, Fonseca E, Clode A, Bartnitzke S, Castedo S and Bullerdiek J. (1998). *Cancer Genet. Cytogenet.*, **101**, 42–48.
- Berner JM, Meza-Zepeda LA, Kools PF, Forus A, Schoenmakers EF, Van de Ven WJ, Fodstad O and Myklebost O. (1997). *Oncogene*, **14**, 2935–2941.
- Bol S, Belge G, Rippe V and Bullerdiek J. (2001). *Cytogenet. Cell Genet.*, **95**, 189–191.
- Bol S, Belge G, Thode B, Bartnitzke S and Bullerdiek J. (1999). *Cancer Genet. Cytogenet.*, **114**, 75–77.
- Bondeson L, Bengtsson A, Bondeson AG, Dahlenfors R, Grimelius L, Wedell B and Mark J. (1989). *Cancer*, **64**, 680–685.
- Bullerdiek J, Bösch C and Bartnitzke S. (1987). *Cancer Genet. Cytogenet.*, **24**, 205–212.
- Dal Cin P, Sneyers W, Aly MS, Segers A, Ostijn F, Van Damme B and Van den Berghe H. (1992). *Cancer Genet. Cytogenet.*, **60**, 99–101.
- Fedele M, Berlingieri MT, Scala S, Chiariotti L, Viglietto G, Rippel V, Bullerdiek J, Santoro M and Fusco A. (1998). *Oncogene*, **17**, 413–418.
- Feinberg AP and Vogelstein B. (1983). *Anal. Biochem.*, **132**, 6–13.
- Kievits T, Dauwerse JG, Wiegant J, Devilee P, Breuning MH, Cornelisse CJ, Van Ommen GJ and Pearson PL. (1990). *Cytogenet. Cell Genet.*, **53**, 134–136.
- Rippe V, Belge G, Meiboom M, Kazmierczak B, Fusco A and Bullerdiek J. (1999). *Genes Chromosomes Cancer*, **26**, 229–236.
- Teyssier JR, Liautaud-Roger F, Ferre D, Patey M and Dufer J. (1990). *Cancer Genet. Cytogenet.*, **50**, 249–263.
- CTTCCCCGATGGATGGT-3' 5'-GGTAGCGGGAGCAAT CACAAAAGTGTAA-3' (*THADA-FUS7p*).
- Amplifications were carried out in a final volume of 50 µl containing 0.5 µl template cDNA, 0.05 U/µl *Taq* DNA Polymerase recombinant (Invitrogen, Karlsruhe, Germany), 0.2 µM of each primer, 200 µM dNTP mix, and 1 × PCR R × N Puffer (Invitrogen, Karlsruhe, Germany). After an initial denaturation at 94°C for 3 min, 35 cycles were performed. Each cycle consisted of denaturation (94°C) for 45 s, annealing for 45 s (58°C, *THADA-FUS3p*; 60°C, *THADA-FUS7p*), and extension for 1 min at 72°C.

#### Northern blot hybridization

Expression patterns of *THADA* were studied using a commercially available Northern blot membrane (Human Endocrine System MTN Blot, Clontech, Palo Alto, CA, USA). A partial cDNA clone of *THADA* was labeled with <sup>32</sup>P by random primer extension (Feinberg and Vogelstein, 1983) and used as molecular probe. The probe was amplified by PCR using primers 5'-AGAGTAAATCCAAACGTGAACCAGAGAATGAGT-3' and 5'-ATTGGGATGAGGCCTTCAGGGGATGA-3'. Hybridization was carried out as described (Rippe *et al.*, 1999), with 30 min prehybridization and hybridization for 1 h at 68°C. The membranes were washed four times for 10 min at room temperature in 2 × SSC/0.05% SDS, and twice for 20 min at 50°C in 0.1 × SSC/0.1% SDS. Signals were visualized by using a STORM phosphorimager 860 (Amersham Pharmacia Biotech, Little Chalfont, England).

The GenBank Accession numbers are: *THADA*, AY149629; *THADA-FUS3p*, AY149630; *THADA-FUS3pA*, AY149631; *THADA-FUS7p*, AY149632; *THADA-A2*, AY149633; death receptor-interacting protein mRNA, AF323176; BAC clones from RPC-11: 1069E24, AQ694385, AQ703756; 339H12, AC010883; 183F15, AC092838; 204D19, AC092615; 167M22, AC093174; 379L16, AC079780.

#### Acknowledgements

We thank Jessica Hommes for technical assistance.

# **XV.**

## **The canine *HMGA1* gene maps to CFA 23.**

**Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, Bullerdiek J.**

*Anim Genet.* 2003; 34(1): 68-9.

Contribution to the work

- Planning and coordination of molecular done work
- Radioactive BAC screening
- Assistance at the composing of the publication

markers were identified by linkage analysis using the two-point option in CRIMAP with a threshold value of LOD3.

**Allele frequency:** The PCR reactions were supplemented with 0.6  $\mu$ M fluorescent dUTP [R110] (Applied Biosystems), so that allele size could be determined using either an Applied Biosystems 377 or 3700 automated sequencer running GENESCAN 2.1 software. Table 1 shows the allele sizes that segregate in the two full-sibling families described above and the chromosome location of these markers together with the closest linked marker,<sup>1</sup> recombination fraction and LOD score.

**Acknowledgements:** The Horserace Betting Levy Board and the Childwick Trust supported J.E.S and M.M.B. Awards from the Morris Animal Foundation and the United States Department of Agriculture supported J.R.M and L.J.A.

#### Reference

1 Swinburne J. E. *et al.* (2000) *Genomics* **66**, 123–34.

*Correspondence:* J. E. Swinburne (jswinbur@hgmp.mrc.ac.uk).

### The canine *HMGA1* gene maps to CFA 23

**K. Becker\***, **H. Murua Escobar\***, **A. Richter\***,  
**B. Meyer\***, **I. Nolte<sup>†</sup>** and **Jörn Bullerdiek\***

\*Center for Human Genetics, University of Bremen, Bremen, Germany. <sup>†</sup>Clinic for Small Animals, Hannover School of Veterinary Medicine, Hannover, Germany

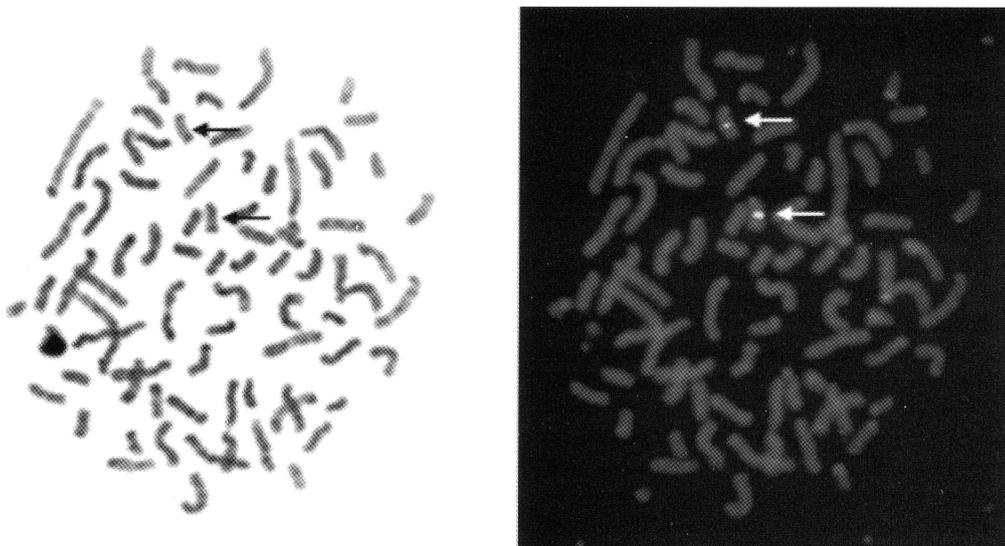
Accepted 26 October 2002

**Introduction:** Parallels between human and canine tumours have often been described. Human chromosomal rearrangements on 6p21 involving the *HMGA1* gene have been described in various benign mesenchymal tumours like pulmonary chondroid hamartomas, uterine leiomyomas, endometrial polyps and lipomas<sup>1,2</sup>. So far, it is not clear if comparable translocations occur in the corresponding canine tumour as well. To further elucidate that question, we have mapped the canine *HMGA1* gene.

**BAC clone and probe:** A *HMGA1* DNA probe was generated by PCR spanning part of the exon 6, the complete intron 6 and part of exon 7 (primer up: AGC GAA GTG CCA ACA CCT AAG AGA/Lo: CCT TGG TTT CCT TCC TGG AGT TG) on DNA derived from the canine cell line MTH52 (Center for Human Genetics, Bremen, Germany), cloned, sequenced and used for screening of a canine BAC library. Filters were obtained from the BAC-PAC RESOURCES/Childrens Hospital, Oakland, CA, USA (<http://www.chori.org/bacpac>). BAC-screening was performed following manufacturers' instructions. To rule out false-positive results, a PCR using the initial primer pair used for the screening probe was performed, cloned and sequenced.

**Fluorescence in situ hybridization:** Metaphase preparations and fluorescence *in situ* hybridization (FISH) were performed as described previously<sup>3</sup>. Ten well-spread metaphases were examined all showing a signal on CFA 23 on both chromatids of both chromosomes CFA 23 (Fig. 1).

**Comments:** Chromosomal rearrangements of HSA 6p21 involving *HMGA1* represent the second most frequent specific translocations in human tumours. The assignment of the canine *HMGA1* gene to CFA 23 clearly shows that the chromosomal region to which the canine *HMGA1* gene has been



**Figure 1** An example of a metaphase spread after fluorescence *in situ* hybridization with signals on both chromosomes 23 (right) and the same metaphase after GTG-banding (left).

mapped, is not a hotspot of chromosomal breakpoints seen in canine tumours. The hotspots that have been found in the dog genome so far, include chromosomes 1, 19 and 25 which are preferentially involved in chromosomal fusions<sup>4</sup>. The X-chromosome of the dog, in contrast, is frequently affected by structural aberrations. Therefore, in contrast to humans, the activation of *HMGAI* as a result of chromosomal translocations does not seem to play a considerable role in canine tumours. This may be due to the fact that the corresponding changes are not able to induce benign tumours in the dog or to stimulate their growth. Alternatively, there may be factors favouring the occurrence of the structural changes in humans which are lacking in dogs.

No homology has been found between human chromosome 6, to which the *HMGAI* is mapped, and canine chromosome 23, rather human chromosome 6 shares homology with canine chromosome 22 and 8<sup>5</sup>. In our FISH studies no metaphase shows signals on these dog chromosomes.

**References**

- 1 Kazmierczak B. *et al.* (1996) *J Natl Cancer Inst* **88**, 1234–6.
- 2 Dal Cin P. *et al.* (1999) *Genes Chromosomes Cancer* **24**, 286–9.
- 3 Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94**, 194–5.
- 4 Reimann N. *et al.* (1999) *J Natl Cancer Inst* **91**, 1688–9.
- 5 Yang F. *et al.* (1999) *Genomics* **62**, 189–202.

Correspondence: Dr J. Bullerdiek (bullerd@uni-bremen.de)

**Contribution of a new set of canine microsatellites to the knowledge of the canine genetic map**

**D. Parra\*, G. Dolf† and S. Dunner\***

\*Laboratorio de Genética Molecular, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.

†Institute of Animal Genetics, University of Berne, Switzerland

Accepted 28 October 2002

*Source/description:* *Canis familiaris* DNA was digested with *Sau3AI* and fragments ranging 700–1000 bp in length were ligated into the *Bam*HI site of a pre-digested and de-phosphorylated pUC18 vector (Amersham Pharmacia Biotech, Amersham, UK) and transformed into *Escherichia coli* SURE<sup>®</sup> cells (Stratagene, La Jolla, CA, USA). A total of 1200 recombinant clones were pooled in groups of 10 and the pools were spotted onto a nylon membrane, screened with digoxigenin-labelled probes (TG)<sub>10</sub> and (AAAT)<sub>7</sub>, and the positive pools were screened individually to isolate the final positive clones. Ten positive clones were sequenced in an ABI-310 sequencer (Applied Biosystems, Foster City, CA, USA) from which six

**Table 1** Features of the microsatellites, including heterozygosity (H), polymorphic information content (PIC)<sup>2</sup>, effective number of alleles (ENA)<sup>3</sup>, number of informative meioses (NIM), linkage group and chromosomal location.

Locus	GenBank Accession no.	Primer sequences (5' → 3')	Repeat unit	Size range (bp)	Annealing T (°C)	Alleles (n)	H	PIC	ENA	NIM (%) <sup>1</sup>	Assigned linkage group	Chromo-some location
UCMCF12	AY059379	GGAGCTCTCGCCAGTATG ATCTGTAGTAAAGCTGAC	(AAAT) <sub>12</sub>	135–151	58	5	51.4	48.1	2.1	78 (36)	L28/33	CFA16
UCMCF40	AF210623	TTTACAACCTAAATGTCCTTG CATTCCITTTTCATGGTTAAT	(GT) <sub>20</sub>	103–121	58	9	67.44	63.5	3.1	110 (51)	L19	CFA5
UCMCF54	AF210621	CATGGAGCCTGCTCTCC GCGCTTCTCCAAGTAACAG	(CT) <sub>13</sub> (GT) <sub>9</sub>	154	50	1	0	0	1	–	–	–
UCMCF71	AF210622	ACTCAAAGCCATCTTGTACA AAGAAGCTGCAGAGCTACG	(AC) <sub>14</sub>	142–156	58	7	69.78	66.9	3.3	69 (32)	L26	CFA1
UCMCF96	AF210620	GGTGGACTTAGAGGATCTGG GAGCAGCAGCCTGGACTAC	(TG) <sub>19</sub> (AG) <sub>14</sub>	225–241	63	8	82.74	80.5	5.8	136 (64)	L18/33	CFA10
UCMCF117	AF448484	GAGGATCCTGGAGTCTCGG CTTGCTTGGAACTAGTACTTGG	(CT) <sub>6</sub>	186	58	1	0	0	1	–	–	–

# XVI.

**The canine *ERBB2* gene maps to a chromosome region frequently affected by aberrations in tumors of the dog (*Canis familiaris*).**

**Murua Escobar H, Becker K, Bullerdiek J, Nolte I**

*Cytogenet Cell Genet.* 2001; 94(3-4): 194-5.

Contribution to the work

- Cloning and verification of the probe used for BAC screening
- Radioactive BAC screening and verification
- Composing of the publication together with Kristina Becker

## The canine ERBB2 gene maps to a chromosome region frequently affected by aberrations in tumors of the dog (*Canis familiaris*)

H. Murua Escobar,<sup>a</sup> K. Becker,<sup>a</sup> J. Bullerdiek,<sup>a</sup> and I. Nolte<sup>b</sup>

<sup>a</sup>Center for Human Genetics, University of Bremen, Bremen;

<sup>b</sup>Clinic for Small Animals, Hannover School of Veterinary Medicine, Hannover (Germany)

**Abstract.** The dog offers an increasingly important model for several human diseases, including cancer. Accordingly, the results of canine gene mapping studies will be of considerable significance. Herein, we have addressed the mapping of the canine gene ERBB2 (alias HER2, NEU). ERBB2 is a proto-oncogene encoding a tyrosine kinase receptor protein, the overexpression of which correlates with a more rapid progression and

a worse prognosis in breast cancer. In addition, it apparently plays a role in the development of other tumors as well. By fluorescence in situ hybridization (FISH), we have mapped the canine ERBB2 to 1q13.1. Cytogenetic studies of canine tumors revealed that this region is very often affected by clonal chromosome aberrations in tumors of the dog.

Copyright © 2002 S. Karger AG, Basel

Although relatively little is yet known about cytogenetic changes in canine tumors, some differences compared to the patterns of cytogenetic deviations in human tumors are emerging. Among these differences is the relatively low frequency of chromosome translocations in canine tumors. On the other hand chromosome fusions with breakpoints close to the centromere often have been described (Reimann et al., 1994). Chromosome 1 in particular is frequently affected by these changes. Its pericentromeric area is so far one of the most prominent hotspots for occurrence of chromosome aberrations in the canine karyotype (Reimann et al., 1999). Fusions of that area with other chromosomes have been described for example by Horsting et al. (1999). Despite the high frequency of these aberrations, so far no attempt has been made to correlate these aberrations to particular genes. Herein, we report on the mapping of the gene encoding the tyrosin kinase receptor protein ERBB2 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; neuro/glioblastoma derived oncogene homolog) to that region.

### Materials and methods

An ERBB2 cDNA probe was used for hybridization of canine RCPI 81 BAC/PAC filter (BACPAC RESOURCES, Childrens Hospital, Oakland, USA). The probe was generated by PCR (Primer Up: CGA CCG GCC GGT GCC ACC CT; Lo: TTC AAA GGT GCT GGG TGG AGA GC) on cDNA of the canine mama cell line MTH52 (Center for Human Genetics, Bremen, Germany) and cloned and sequenced for verification. The probe labeling was performed by random prime labeling (Roche Diagnostics, Mannheim, Germany) as described in the manufacturer's protocol with 50  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia Biotech, Freiburg, Germany). Purification of the labeled probe was done using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech, Freiburg, Germany) and probe was stored at -20 °C before use.

The filters were placed in minimum volume of Church's buffer (0.15 mM BSA, 1 mM EDTA, 0.5 M NaHPO<sub>4</sub>, 7% SDS) and transferred into hybridization bottles. The filters were prehybridized at 65 °C for 1 h in 25 ml Church's Buffer. Hybridization was performed at 65 °C overnight (16–18 h) in the same solution. All further steps were performed according to standard protocols.

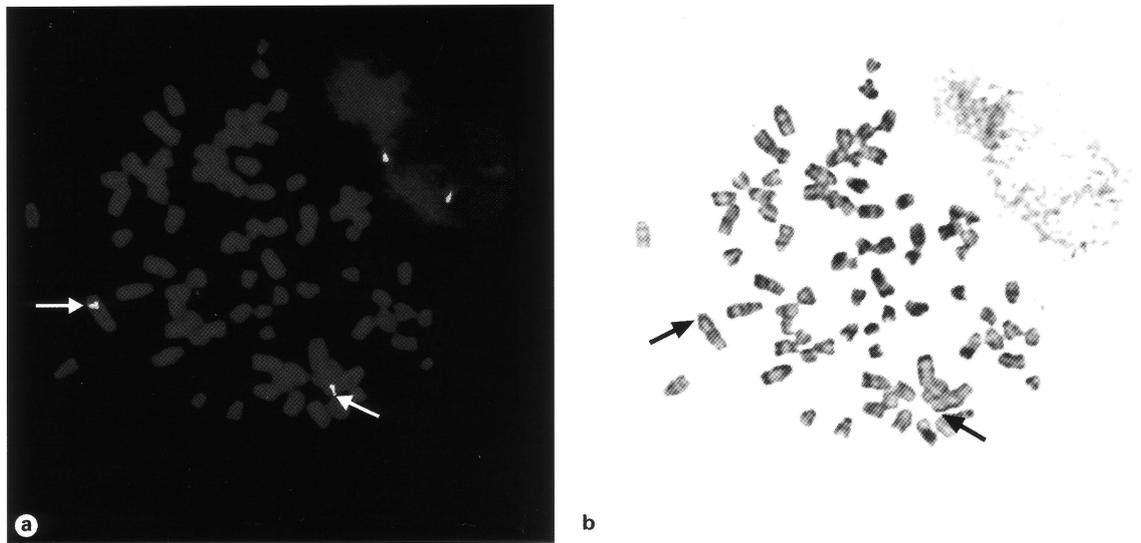
BAC 401-B7 gave a positive signal which was verified by PCR using the initial primer pair used in the screening process. The generated PCR product was separated on a 1.5% agarose gel, recovered, cloned and sequenced.

Metaphase preparations were obtained from blood samples of different dogs. The samples were stimulated with PHA and cultured for 96 h at 37 °C. After a 1.5-hour colcemide (0.1  $\mu$ g/ml) incubation the lymphocytes were harvested according to routine procedures. Prior to FISH chromosomes were stained using the GTG-banding method and metaphases were photographed using a CCD camera. The slides were destained in 70% ethanol for 15 min and air dried.

FISH was performed using the protocol of Fischer et al. (1996) with some modifications. BAC DNA was digoxigenin-labeled (Dig-Nick-Translation-

Received 31 May 2001; revision accepted 21 September 2001.

Request reprints from Dr. J. Bullerdiek, Center for Human Genetics,  
University of Bremen, Leobener Str. ZHG, D-28359 Bremen (Germany);  
telephone: 0049 421 218 4239; fax: 0049 421 218 4239;  
e-mail: bullerd@uni-bremen.de



**Fig. 1.** An example of a metaphase spread after FISH with signals on both chromosomes 1 (a) and the same metaphase after GTG-banding (b).

Kit, Roche Diagnostics). The hybridization-mixture contained 125–175 ng probe, 43.2 pg salmon sperm DNA, 1000–1200 ng sonicated dog DNA, 2× SSC, 2× SSPE, 50% formamide and 10% dextran sulfate. The chromosomes were stained with propidium iodide. Chromosome G-bands were identified according to Reimann et al. (1996).

### Results and discussion

Sixteen well spread metaphases were examined and all showed a signal on 1q13 on both chromatids of both chromosomes 1 (Fig. 1). Yang et al. (1999) found no homology between human chromosome 17 where ERBB2 maps and canine chromosome 1. According to Yang et al. (1999) there is homology of human chromosome 17 with canine chromosomes 5 and 9. Our FISH studies showed no signals on these chromosomes.

During the past few years, the dog has become an interesting model organism for several human diseases and tumors. Aberrations in tumors of the dog involving chromosome 1 were described earlier by several authors (Bartnitzke et al., 1992; Mayr et al., 1993; Reimann et al., 1998; Horsting et al., 1999) in various tumors of the dog such as leukemias (Reimann et al., 1998), melanomas (Horsting et al., 1999) and breast cancer (Bartnitzke et al., 1992). However, the assignment of genes, especially oncogenes, to that particular chromosome has not been demonstrated yet. The region to which the canine ERBB2 has been mapped is one of the most frequently rearranged chromosome regions in canine tumors (Reimann et al., 1999). Translocations of that region may lead to an activation of that gene. On the other hand, trisomy 1 is also described by different authors (Mayr et al., 1991; Reimann et al., 1998) and could lead to an overexpression of genes such as ERBB2 as well.

### References

- Bartnitzke S, Motzko H, Caselitz J, Kornberg M, Bullerdiek J, Schloot W: A recurrent marker chromosome involving chromosome 1 in two mammary tumors of the dog. *Cytogenet Cell Genet* 60:135–137 (1992).
- Fischer P, Holmes N, Dickens H, Thomas R, Binns M, Nacheva E: The application of FISH techniques for physical mapping in the dog (*Canis familiaris*). *Mammal Genome* 7:37–41 (1996).
- Horsting N, Wohlsein P, Reimann N, Bartnitzke S, Bullerdiek J, Nolte I: Cytogenetic analysis of three oropharyngeal malignant melanomas in dogs. *Res Vet Sci* 67:149–151 (1999).
- Mayr B, Gilli H, Schleger W, Reifinger M, Burtscher H: Cytogenetic characterization of mammary tumors in two domestic dogs. *Zentralbl Veterinärmed A* 38:141–147 (1991).
- Mayr B, Eschborn U, Loupal G, Schleger W: Trisomy 1 in a canine mammary tubular adenocarcinoma complex type. *Vet Pathol* 30:311–313 (1993).
- Reimann N, Rogalla P, Kazmierczak B, Bonk U, Nolte I, Grzonka T, Bartnitzke S, Bullerdiek J: Evidence that metacentric and submetacentric chromosomes in canine tumors can result from telomeric fusions. *Cytogenet Cell Genet* 67:81–85 (1994).
- Reimann N, Bartnitzke S, Bullerdiek J, Schmitz U, Rogalla P, Nolte I, Ronne M: An extended nomenclature of the canine karyotype. *Cytogenet Cell Genet* 73:140–144 (1996).
- Reimann N, Bartnitzke S, Bullerdiek J, Mischke R, Nolte I: Trisomy 1 in a canine acute leukemia indicating the pathogenetic importance of polysomy 1 in leukemias of the dog. *Cancer Genet Cytogenet* 101:49–52 (1998a).
- Reimann N, Nolte I, Bullerdiek J: Cancer genetics going to the dogs: The future has begun. *J natl Cancer Inst* 91:1688–1689 (1999).
- Yang F, O'Brien PC, Milne BS, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D, Ferguson-Smith MA: A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. *Genomics* 62:189–202 (1999).

## 8 Danksagung

Prof. Dr. Jörn Bullerdiek möchte ich in erster Linie für das Vertrauen danken, welches er 2000 in mich setzte, als das "Hundeprojekt" vor dem Ende stand. Des Weiteren möchte ich ihm natürlich für die Möglichkeit danken, diese Arbeit in seinem Institut zu realisieren sowie für die Überlassung des Themas, die vielen Anregungen und die Diskussion der Arbeit.

Prof. Dr. Ingo Nolte danke ich vor allem für die Möglichkeiten, die er mir eröffnet und zur Verfügung gestellt hat. Weiterhin danke ich ihm sehr für die viele Zeit, die er sich, trotz seines vollen Terminkalenders, immer für mich eingeräumt hat. Für die Übernahme des Koreferats dieser Arbeit nochmals vielen Dank.

Ein ganz herzliches „DANKE MANN“ meinem „Team“ (*in no particular order*): Britta „BriDDy“ Meyer, Andreas „...kannst Du mal kommen mein Computer...“ Richter, Jan „...mach es einfach so..!“ Thies Soller, Kathrin „Günni“ Günther, Kristina (nun Mutter) Becker, dem „Susannchen“ Susanne Winkler, Jan D. Sperveslage „Regel Nr.1: Kein Testosteron im Labor“ und Katharina „dat Katilein“ Sterenczak. Ohne Euch wäre dies nie zustande gekommen.

Des weiteren Rolf Nimzyk für die sehr hilfreichen bioinformatischen Analysen und Janina Meyer für die Zusammenarbeit.

Dem gesamten ZHG-Team, welches auf die eine oder andere Weise immer für Unterhaltung gesorgt hat - nochmals vielen Dank.

Dem gesamten TiHo-Team vielen Dank für die direkte und indirekte Unterstützung. Besonders Nina Eberle, die mit Engelsgeduld allen Sonderwünschen nachgekommen ist.

Für den vielen Spaß und das gute Teamwork noch mal ein DANKE an Sven Hauke, Aljoscha M. Flohr, Cornelia Blank, Corina Rohen-Bullerdiek, Inga Lemke, Heike Munzinger und Claudia Schlüter.

Ooohhhh es geht auf Seite 2 zu.....

Lars Borrmann (*irgendwann werd ich Dich im Squash schlagen*) einen ganz besonderer Gruß und Dank, denn als das „Dunkle Zeitalter“ kam, wurden die Freunde rar.

Ich danke an dieser Stelle noch mal ganz herzlich und besonders meiner Freundin Dr. Maren Meiboom, die trotz Susannes ständiger Nachfragen („sag mal.. ist der eigentlich immer so?“), es all die Zeit mit mir ausgehalten hat und die wahre Natur des Havana Club in zahllosen Selbstversuchen ergründet hat. Ach ja.. und für das Teamwork und so....

Einen besonderen Gruß an meine Freunde, die die Höhen und Tiefen der Entstehung dieser Arbeit zwangsweise miterlebt haben: Maurice, Ingo, Crash T., Hajo, Stössel, Puffel, Minks, Spink, Felix, Kai, Scheibe, deren jeweiligen Freundinnen und dem Rest.

Meinem Vater Hugo Murua Chevesich vielen Dank dafür, dass er mich zu dem gemacht hat was ich heute bin und der mir beibrachte, wie er sagen würde: „...den Dingen die Bedeutung zu geben, die sie wirklich haben.“

Dem Rest der Familie.. DANKE... und am Ende das wichtigste...:

***Hi MAM.....!!!!***