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Analysis of the Genome Stability of Uterine Fibroids with Different Degree of *HMGA* Expression

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Herewith, I declare that this thesis is a presentation of my original research work on: “Analysis of the Genome Stability of Uterine Fibroids with Different Degree of *HMGA* Expression“. Wherever contributions of others are involved, every effort is made to indicate it clearly, with due reference to the literature, and acknowledgment of collaborative research and discussions.

Maliheh Hashemi Nezhad,
born in may 2nd, 1975, Yazd-Iran

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List of abbreviations

A	Adenine
A/A	Aberrant/Aberrant
A/A/A	Aberrant/Aberrant/Aberrant
A/A/N	Aberrant/Aberrant/Normal
A/N	Aberrant/Normal
BMI	Body Mass Index
C	Cytosine
cdna	copy DNA
chrb	chromosome break
chtb	chromatid break
Cp	Composite
C-Terminal	Carboxy-Terminal
Del	Deletion
DNA	Deoxyribonucleic Acid
EDTA	Ethylendiamintetraacetat
ES cell	Embryonic Stem cell
ESR2	Estrogen Receptor Beta Gene
FFPE	Formalin-Fixed, Paraffin-Embedded
FISH	Fluorescence In Situ Hybridization
G	Guanine
G6PD	Glucose 6- Phosphate Dehydrogenase
GTG banding	G-banding with Trypsin and Giemsa
HMGA	High Mobility Group AT-hooks protein
HMGA1	High Mobility Group AT-hooks 1 gene
HMGA1	High Mobility Group AT-hooks 1 protein
HMGA2	High Mobility Group AT-hooks 2 protein
HMGA2	High Mobility Group AT-hooks 2 gene
HOPE	Hepes-glutamic acid buffer mediated Organic solvent Protection Effect
ISCN	International System for Human Cytogenetic Nomenclature
LCS	Let-7 Complementary Sites
LMS	Leiomyosarcoma

M	Mosaic
M-MLV	Moloney-Mause-Leukemia-Virus
MMP-2	Matrix Metalloproteinase-2
mRNA	messenger Ribonucleic Acid
NTC	Nontemplate Controls
N.M	Non-Mosaic
NHEJ	Nonhomologous End Joining
OIS	Oncogene-Induced Senescence
P	Passage
P	*P-value*
p	p arm of chromosome
PBS	Phosphate buffered saline
PCH	Pulmonary Chondroid Hamartomas
PCR	Polimerase Chain Reaction
q	q arm of chromosome
RNA	Ribonucleic Acid
RNase	Ribonuklease
Rpm	Rounds per minute
rRNA	ribosomal RNA
RT	Reverse Transkription
UL	Uterine Leiomyomas
UTR	Untranslated Region

Summary

Uterine leiomyomas (UL), as the most common gynecologic tumors in women, with a mesenchymal origin, are attended scientifically due to their high prevalence, irrecoverable complications, and their high therapeutic burden. Genetic factors are among the factors playing an important role in the initiation of these tumors.

This thesis aims at the cytogenetic molecular investigations in a large series of UL (N=261), aimed to a better understanding of the correlation between chromosomal changes and the expression of related genes, as well as the genome instability in correlation with these genes. This work is divided into: chromosome analyses, molecular tests including quantification of the expression of *HMGA* genes by using real-time quantitative RT-PCR, long-term *in vitro* expansion of myoma cells, and survey of genome stability in UL.

This study has discovered that:

From a total of 261 UL, 29.1% showed an abnormal karyotype by conventional cytogenetic techniques. The abnormalities are correlated with tumor size and an overexpression of *HMGA* genes in two major cytogenetic subgroups.

HMGA2 was overexpressed in the myomas with 12q14~15 rearrangements in comparison with matched myometrium and cytogenetically normal tumors.

The overexpression of *HMGA2* was also observed in normal UL compared with myometrial tissue that shows a general role of *HMGA2* overexpression in the pathogenesis of UL.

Expression of *HMGA1* was observed in a higher level in the myomas with 6p21 aberrations than that in myometrium and normal UL.

Increase of the average expression of *HMGA1* mRNA in UL with 6p21 rearrangements was much less stronger than that of *HMGA2* mRNA in case of 12q14~15 aberrations.

Long-term culture of myoma cells with different levels of HMGA proteins did not result in immortalization of these cells, even the existence of a high proliferative rate in the cells with an overexpression of *HMGA2*.

Based on the investigations concerning genome stability in UL, no correlation between HMGA2 level and the increase of genomic instability was shown. This raises doubts about a protection maybe by other factors accompanied with a high HMGA2.

Further studies are still recommended in this area. Concerning the high homology of HMGA2 and HMGA1 proteins, it seems possible that HMGA1 leaves more effects on the developements of UL.

Nevertheless, it is suggested to do more detailed analyses of the HMGA1 gene expression and protein levels. Moreover, further investigations on other factors involved in cell growth and correlated with HMGA proteins are necessary.

Zusammenfassung

Uterusleiomyome (UL), als die häufigsten gynäkologischen Tumoren bei Frauen, mit einem mesenchymalen Ursprung, sind aufgrund ihres häufigen Vorkommens, ihrer schwer behandelbaren Komplikationen und ihres hohen therapeutischen Aufwandes ins Interessenfeld der Wissenschaft gerückt. Genetische Faktoren spielen unter anderen Faktoren eine sehr wichtige Rolle bei der Entstehung dieser Tumoren.

Ziel dieser Arbeit sind zytogenetische und molekulare Untersuchungen an einer großen Anzahl von UL (N = 261), um sowohl ein besseres Verständnis des Zusammenhangs zwischen den chromosomalen Veränderungen und der Expression der betroffenen Gene, *HMGA1* und *HMGA2,* als auch den Zusammenhang zwischen der Genominstabilität und denselben Genen zu ermöglichen. Diese Arbeit gliedert sich in: Analyse der Chromosomen, molekulare Untersuchungen einschließlich der Quantifizierung der Expression von *HMGA* Genen mit Hilfe von quantitativer Real-Time RT-PCR, Langzeitkultivierung von Myomzellen in vitro und Untersuchung der Genomstabilität in UL.

Diese Studie hat Folgendes herausgefunden:

Unter Verwendung konventionellen zytogenetischen Techniken zeigten 29,1% aller untersuchten UL einen aberranten Karyotyp. In zwei wesentlichen zytogenetischen Subgruppen stehen die Anomalien in Korrelation mit der Tumorgröße und der Überexpression von *HMGA* Gene.

HMGA2 zeigte in den Myomen mit 12q14~15 Rearrangement eine Überexpression im Vergleich zu den dazugehörigen Myometrien und zytogenetisch normalen Tumoren. Die Überexpression von *HMGA2* wurde auch im normalen UL gegenüber Myometrien beobachtet. Dies zeigt, dass die *HMGA2* Überexpression eine besondere Rolle in der Pathogenese der UL spielt.

Die Expression von *HMGA1* erschien in den Myomen mit 6p21 Aberrationen stärker als in den dazugehörigen Myometrien und normalen UL.

Der Anstieg durchschnittlicher Expression von *HMGA1* mRNA in UL mit 6p21 Rearrangierungen war viel weniger stark als der des *HMGA2* mRNA bei 12q14~15 Aberrationen.

Langzeitkulturen der Myomzellen mit unterschiedlichen HMGA Proteingehalt führten nicht zur Immortalisierung dieser Zellen, auch nicht dann, wenn in den Zellen mit *HMGA2*-Überexpresion eine hohe Proliferationsrate vorlag.

Aus den Untersuchungen zur Genomstabilität in UL, konnte keine Korrelation zwischen *HMGA2* Expression und der Zunahme der Instabilität des Genoms festgestellt werden. Dies legt die Vermutung nahe, dass die Genomstabilität durch andere Faktoren unterstützt wird, die einer hohen *HMGA2*-Expression begleiten.

Weitere Studien sind in diesem Bereich noch zu empfehlen. Hinsichtlich der hohen Homologie der *HMGA2* und *HMGA1* Proteinen, scheint eine größere Auswirkung von *HMGA1* auf die Entwicklungen von UL möglich.

Auf jeden Fall sind detailliertere Analysen der *HMGA1*-Expression und Untersuchung des Proteingehalts zu empfehlen. Darüber hinaus sind weitere Untersuchungen von anderen Faktoren notwendig, die eine Rolle beim Zellwachstum spielen und mit HMGA Proteinen zusammenhängen.

1. Introduction

Uterine leiomyomas (UL) are the most common benign tumors of the female genital tract. Although progression of leiomyoma to malignant leiomyosarcoma (LMS) happens very rarely (frequency of <0.1%) (Sandberg, 2005), considering the frequency and complications of this tumor makes it necessary to do further investigations into the etiology of the UL. This study concentrates on understanding of the pathobiology of the UL based on cellular and molecular analysis of myomas.

The reported prevalence of UL is ranging from 3.3% to as high as 87% (Borgfeldt and Andolf, 2000; Baird et al., 2003a). They are common in reproductive age (Kane, 2002). The available studies indicate that at least one third of women over 30 years old have one or more UL (Cramer and Patel, 1990; Baird et al., 2003a; Heinemann et al., 2003). Myomas are more prevalent in African Americans (16-87%) than in European American or European women of reproductive age (9-78%), (Marshall et al., 1997; Chen et al., 2001; Faerstein et al., 2001; Baird et al., 2003a). Furthermore, African American women are diagnosed at earlier ages. Additionally African Americans have more severe symptoms, larger in size and greater in number of tumors than white women (Kjerulff et al., 1996).

Most commonly UL develop within the uterus. However, leiomyomas are also found in other parts of the body, for example in gastrointestinal tract or within the walls of vessels (Blake, 2007). Less frequently UL occur in the cervix and in the fallopian tubes and even less commonly in the broad ligament (Blake, 2007). According to their location in the uterus, UL are classified into three groups: submucous, subserous, and intramural (Kane, 2002). Submucous myomas are located immediately beneath the endometrial or decidual surface of the uterine cavity. Subserous tumors project out from the peritoneal surface of the uterus while intramural forms are confined to the myometrium. Submucous and subserous myomas may at times be attached to the uterus by stalks (pedunculated).

Histopathological analyses reveal that UL are well circumscribed, pseudoencapsulated, solid and pearly white or lightly tanned round masses with size usually ranging from 1 mm to >30 cm (Blake, 2007), although a diameter as large as 72 cm was documented too (Kane, 2002). Microscopically UL are showing interlacing bundles of spindle-shaped or stellate-type smooth-muscle cells with little cellular

pleomorphism or mitotic activity (<5/10 hpf). The growth pattern of UL shows a low mitotic activity (Blake, 2007).

Since the majority of tumors are asymptomatic, many remain undiagnosed (Cramer and Patel, 1990). The most common non-invasive method of diagnosis is transvaginal ultrasound. The clinical symptoms of UL include excessive menorrhagia, severe abdominal pain, urinary incontinence, frequent urination, backache, and constipation (Carlson et al., 1994; Kjerulff et al., 1996; Coronado et al., 2000; Morton, 2000; Ligon and Morton, 2001; Kane, 2002; Wegienka et al., 2003; Sandberg, 2005). The severity of clinical symptoms depends on the location of the UL within the uterus (Cotran et al., 1989) and their size and number (Buttram and Reiter, 1981). Leiomyomas may lead to serious complications e.g. infertility, spontaneous abortions, premature labor, dystocia or anemia (Carlson et al., 1994; Greenberg and Kazamel, 1995, Kjerulff et al., 1996; Morton, 2000; Coronado et al., 2000; Kane, 2002; Wegienka et al., 2003).

The etiology of myoma is largely unknown; nevertheless, several risk factors have been indicated in epidemiologic studies (see Tab. 1.1).

Treatment alternatives include hormonal therapy (anti-estrogen or anti-progesterone), observation, embolization, myomectomy, or hysterectomy (Kane, 2002). Choice of treatment depends on various factors, including size of tumor, severity of symptoms, and rate of growth, as well as the woman's desire to have children (Buttram and Reiter, 1981).

Many hypotheses have been advanced to explain the etiology of the UL through the investigation of the hormonal factors, growth factors, genetic factors, and molecular biology. It has been suggested that an increased level of estrogen and progesterone lead to an increment of the mitotic rate that may contribute to myoma formation by increasing the risk of somatic mutations (Rein, 2000). Other hypotheses based on the findings of significantly increased levels of estrogen receptor in the myometrium of those who develop myomas suggest a correlation between the pathogenesis of UL and an inherent abnormality in the myometrium (Richards and Tiltman, 1996).

Furthermore, Dixon et al., (2002) suggested that the most significant contributor to growth of UL (independent of tumor size and also autonomously for each tumor in a given patient) is abnormal cell proliferation (Dixon et al., 2002). Others have suggested a predisposing genetic factor for UL on the basis of its familial and ethnic

predominance (Marshall et al., 1997; Schwartz et al. 2000a; Van Voorhis et al., 2002; Okolo et al., 2005; Uimari et al., 2006).

Tab.1.1: Potential risk factors associated with uterine leiomyoma.

Characteristic/Factor	Risk associated with myoma	References
African American ethnic group	↑	Marshall et al., 1997; Chen et al., 2001; Faerstein et al., 2001; Baird et al., 2003a
Late reproductive age (>30)	↑	Schwartz, 2001; Wise et al., 2005; Baird, 2005
Early menarche	↑	Schwartz, 2001; Flake et al., 2003
Nulliparity	↑	Myers et al., 2002; Flake et al., 2003; Baird et al., 2003a; Wise et al., 2005
Tamoxifen treatment	↑	Deligdisch, 2000
Pregnancy	↓	Parazzini et al., 1988; Marshall et al., 1998
Multiparity	↓	Kjerulff et al., 1996; Parazzini et al., 1996; Luoto et al., 2000; Myers et al., 2002
Menopause	↑↓	Schwartz, 2001; Palomba et al., 2002; Flake et al., 2003
Oral contraceptive	↑↓	Ross et al., 1986; Parazzini et al., 1988; Marshall et al., 1998; Schwartz, 2001; Faerstein et al., 2001; Myers et al., 2002; Flake et al., 2003; Wise et al., 2004
BMI	↑↓	Parazzini et al., 1988; Marshall et al., 1998; Luoto et al., 2000; Schwartz, 2001; Myers et al., 2002; Flake et al., 2003; Brett and Higgins, 2003
Hypertension	↑↓	Schwartz, 2001; Palomba et al., 2002; Flake et al., 2003
Smoking	↑↓	Ross et al., 1986; Cramer et al., 1995; Marshall et al., 1998; Schwartz, 2001; Faerstein et al., 2001; Wise et al., 2004
Diet	↑↓	Woods et al., 1996; Chiaffarino et al., 1999
Abortion	↑↓	Parazzini et al., 1996,1988
Perineal talc use	↑↓	Parazzini et al., 1988; Marshall et al., 1998; Faerstein et al., 2001
History of pelvic inflammatory disease	↑↓	Parazzini et al., 1988; Marshall et al., 1998; Faerstein et al., 2001
Chlamydial infection	↑↓	Parazzini et al., 1988; Marshall et al., 1998; Faerstein et al., 2001
Use of intrauterine device	↑↓	Parazzini et al., 1988; Marshall et al., 1998; Faerstein et al., 2001

↑: increased probability of UL, ↓: decreased probability of UL, ↑↓: inversely associated with myoma in different studies.

The possibility of genetic predisposition to fibroids remains an unanswered question. So far the issue of genetic predisposition has been investigated from four perspectives: twin studies, familial aggregation, ethnic predisposition, and association with an inherited syndrome (Winkler and Hoffmann, 1938, Thyresson and Su, 1981; Kurbanova et al., 1989; Treloar et al., 1992; Vikhlyaeva et al., 1995; Marshall et al., 1997; Baird et al. 1998; Luoto et al., 2000; Schwartz et al. 2000a,b, Chen et al., 2001; Faerstein et al., 2001; Gross and Morton, 2001; Baird et al., 2003a). The available studies on the genetic predisposition show higher correlation for hysterectomy in monozygotic than dizygotic twins (Treloar et al., 1992, Luoto et al., 2000), although, by ultrasound examination the risk ratio for fibroids in a monozygous twin whose sister had been diagnosed with fibroids was the same as for a dizygous twin (Luoto et al., 2000). That could be because of low participation rate (Flake et al., 2003); higher occurrence of myomas in first-degree relatives of women with UL than those without (Winkler and Hoffmann 1938, Kurbanova et al. 1989, Vikhlyaeva et al., 1995, Schwartz et al. 2000a); higher prevalence among African American women than other ethnic groups and also higher clinical prevalence because of a higher frequency of multiple lesions and greater size of the fibroids (Marshall et al., 1997; Baird et al. 1998; Chen et al., 2001; Faerstein et al., 2001; Baird et al., 2003a); and existence of a family histories in Reed's Syndrome or multiple leiomyomata in the skin, uterus, or both (Fisher and Helwig 1963; Reed et al. 1973; Thyresson and Su, 1981).

There is a general acceptance in the literature that UL are monoclonal. Studies supporting this acceptance have been based on the Lyon hypothesis that only one X chromosome is active in every female cell. Thus, some studies of clonality used the X-linked glucose 6- phosphate dehydrogenase (G6PD) isoenzymes (Linder and Gartler, 1965; Townsend et al., 1970). Other studies analyzed the existence of both G6PD types (A and B) in almost all myometrium samples in contrast to only one G6PD type in each of UL, also identifying both tumors in the same patient. Further investigations to discriminate between active and inactive alleles of X-linked genes have used the X-linked androgen receptor gene (Mashal et al., 1994) and the X-linked phosphoglycerokinase gene (Hashimoto et al., 1995).

As for genetic conditions of UL at least four major cytogenetic subgroups have been described:

At least one-half of UL appears to be cytogenetically normal. Abnormality in the cytogenetic of UL is reported in a range of 7% to 46% (Nilbert et al., 1990, Vanni et al., 1991, Kiechle-Schwarz et al., 1991, Pandis et al., 1991, Meloni et al., 1992, Rein et al., 1998, Brosens et al., 1998, Hennig, 1999, Kataokaa et al., 2003).

One of the most common abnormalities is a particular rearrangement of the long arm of chromosome 12 i.e. a translocation between chromosomes 12 and 14, usually as a t(12;14)(q14~q15;q23~q24), with a frequency of 17-46% of karyotypically abnormal UL (Nilbert and Heim, 1990; Hennig, 1999; Ligon and Morton, 2000). This abnormality is of particular interest because the same region of 12q is also commonly rearranged in a variety of other mesenchymal solid tumors e.g. lipomas, breast fibroadenomas, endometrial polyps, pulmonary chondroid hamartomas (PCH), hemangiopericytomas, angiomyxomas (Turc-Carel et al., 1986; Bullerdiek et al., 1997; Calabrese et al., 1991; Mandahl et al., 1993; Vanni et al., 1993; Ozisik et al., 1994; Dal Cin et al., 1995; Fletcher et al., 1995). The critical gene that located in the chromosome 12q14~q15 region is *HMGA2* (Ashar et al., 1995; Schoenmakers et al., 1995). The *HMGA2* expression has been detected in UL with 12q14~15 rearrangements, but not in matched normal myometrium (Gattas et al. 1999). In addition, the 14q23~q24 region is also involved in several mesenchymal benign tumors, including uterine leiomyomas, pulmonary chondroid hamartomas, and endometrial polyps (Walter et al., 1989; Nilbert and Heim, 1990; Rein et al., 1991; Dal Cin et al., 1993, 1995; Fletcher et al., 1995; Vanni et al., 1995; Kazmierczak et al., 1995, 1996). Two candidate genes in this region of chromosome 14 are *ESR2* (Estrogen Receptor Beta Gene) (Pedeutour et al., 1998) and *RAD51L1* (a member of the *RAD51* recombination repair gene family) (Albala et al., 1997; Schoenmakers et al., 1999).

Deletions of the long arm of chromosome 7 are present in some studies as the most frequent abnormality in UL (Nilbert and Heim, 1990; Pandis et al., 1991; Meloni et al., 1992) and by the others as the second most common rearrangement in this tumor (Rein et al., 1991; Vanni et al., 1991; Ligon and Morton, 2000). del(7)(q22q32) is present in about 17-24% of karyotypically abnormal UL (Nilbert and Heim, 1990; Hennig, 1999; Ligon and Morton, 2000).

Another cytogenetic subgroup is characterized by aberration of 6p21, where the gene for another member of *HMGA* family, *HMGA1* is located (Friedmann et al., 1993). Rearrangements include deletions, inversions, translocations, and insertions,

occurring with a frequency of <5-13% (Nilbert and Heim, 1990; Hennig, 1999; Ligon and Morton, 2000). In a small series of UL, Tallini et al. (2000) were able to show that rearrangements of 6p21 leads to an overexpression of *HMGA1*. However, quantifying the expression of HMGA1 mRNA in UL of this subtype was not performed.

Trisomy 12 as another cytogenetic subgroup was reported in as many as 2-12% of karyotypically abnormal UL (Nilbert and Heim, 1990; Hennig, 1999).

Other cytogenetic abnormalities have been reported in UL as well, but with lower frequency. Among these are cases of structural rearrangements involving chromosome 1 particularly in the form of ring chromosomes, also translocations and deletions (Nilbert et al., 1988; Casartelli et al., 1989; Vanni et al., 1989; Havel et al., 1989; Mark et al., 1989), aberrations of the X chromosome preferentially involving Xp11~p22 (Turc-Carel et al., 1988; Vanni et al., 1989; Fan et al., 1990; Mark et al., 1990; Nilbert and Heim, 1990; Ozisik et al., 1992), rearrangements of chromosome 10 including deletions affecting the long arm of chromosome 10 (especially band q22) as well as monosomy 10, and rearrangements of chromosome 3 (Nilbert et al., 1990).

Some studies suggest that the chromosomal rearrangements are in fact secondary events during the development of UL. The secondary nature of the chromosomal rearrangements is supported by the findings suggesting that mosaic karyotype (normal/aberrant) myomas are of the monoclonal origin (Mashal et al., 1994); mosaic karyotype myomas (normal/aberrant) are significantly smaller in size than those solely composed of abnormal cells (Rein et al., 1998).

A correlation between chromosomal anomalies and the size of tumors was found. The mean diameter of myoma among specimens with abnormal (non-mosaic) karyotypes was significantly greater than myomas with normal karyotypes (Rein et al., 1998). In addition**,** Hennig et al. concluded that it is true for myomas with 12q14~15 changes but not for the groups of tumors characterized by deletion of chromosome 7 (Hennig et al., 1999). Brosens et al. (1998) showed a positive relationship between the presence of a cytogenetic abnormality and the anatomic location of a tumor. Submucous myomas had significantly fewer clonal abnormalities (12%) than subserosal (29%) or intramural myomas (35%).

HMGA genes are involved in two major subgroups of karyotypically abnormal UL. *HMGA1* (formerly known as *HMGI(Y)*) is located at chromosomal band 6p21 (in 6p

aberrant group) and *HMGA2* (formerly known as *HMGI-C*) is located at chromosomal band 12q14~15 *(*in 12q14~15 aberrant group).

The high mobility group (HMG) proteins are architectural components affecting the structure and activity of the chromatin because of their ability to bind it (Grosschedl et al., 1994). The higher levels of HMG proteins cause irregular expression of different genes, thus inducing developmental abnormalities. Therefore, HMG proteins are the underlying reason of many diseases and tumorigenesis e.g. due to their up-regulation of *CCNB2* gene by HMGAs in pituitary adenomas (De Martino et al., 2009), inhibition of nucleotide excision repair in breast cancer cells (Adair et al., 2005), or induction of type 2 diabetes, due to an HMGA-dependent downregulation of the insulin receptor (Foti et al., 2005).

HMGA (AT-hooks) proteins which are present in stem cells and casually linked to their self-renewal ability are: able to bind to the minor groove of AT-rich stretches in DNA with three DNA-binding domains; induce conformational changes in chromatin structure and enable the regulation of the expression of various target genes. They can also interact with other proteins by means of acidic C-terminal tail (Fusco and Fedele, 2007). HMGAs are found abundant in undifferentiated and proliferating cells of early embryos and undetectable in fully differentiated cells. In particular, expression of *HMGA2* mRNA has not been detected in any of the several adult tissues tested by RT-PCR (Rogalla et al., 1996) with one interesting exception: expression of HMGA2 during spermatogenesis in testis, at least in mice (Chieffi et al., 2002). Conversely, *HMGA1* is expressed at very low constant levels in normal adult tissues (Chiappetta et al., 1996).

These chromatin binding proteins regulate key signalling pathways in cell proliferation or apoptosis (Sgarra et al., 2004; Hoyos et al., 2004; Fedele et al., 2005). HMGA2 is established as a regulator of human genes linked to human embryonic stem cell growth, mesenchymal cell differentiation and adipogenesis (Li et al., 2007). This protein (HMGA2) is also involved in epithelial-mesenchymal transition by employment of TGF-β (Thuault et al., 2006). Narita et al. (2006) introduced a novel role for HMGA proteins - which are also active in tumor suppressor networks by having a role in cellular senescence and heterochromatin formation. In other independent studies HMGA2 was also identified as a target for the let-7 family of microRNAs (Shi et al., 2009; Rahman et al., 2009; Guoying et al., 2009). The findings based on the analysis of the tumor cell proliferation and cell transformation,

repression of HMGA2 following the disruption of let-7 (Lee and Dutta, 2007; Mayr et al., 2007) support again the oncogenic role of HMGA2.

A decrease of HMGA2 has been recently linked to self-renewal of hematopoietic as well as neural stem cells (Nishino et al., 2008). Overexpression of *HMGA* directly induce a faulty expression of many genes in different benign and malign human tumors e.g. up-regulation of *MMP-2* (Matrix Metalloproteinase-2) gene by HMGA1 in large-cell lung cancer (Hillion et al., 2009).

Other recent studies have linked HMGA proteins with an increased genomic instability due to a reduced capacity to repair DNA damage. Inhibition of nucleotide excision repair by HMGA1 (Adair et al., 2005) and suppression of nonhomologous end joining (NHEJ) repair by overexpression of HMGA2 (Li et al., 2009) are reported as the pathways leading to increased DNA instability. Li et al., (2009) analyzed the cytogenetic stability of fibroblasts transfected by a construct encoding HMGA2 as a hallmark of deficient NHEJ. They introduced HMGA2 as a regulator of NHEJ that impairs DNA-PK dynamics by altering Ku (a heterodimer with DNA end binding activity and necessary for proper DSB repairing by NHEJ (Moore et al., 2005)) binding to DNA ends. On the basis of this analysis Li et al. argued that this regulator, as an inhibitor of NHEJ through reduction of DNA stability, facilitates the accumulation of chromosomal aberrations, a central feature of tumorigenesis.

At the same time, HMGA2 is a protein abundantly expressed during embryonic and fetal life (Rogalla et al., 1996; Li et al., 2007) and it is unlikely that this protein *per se* destabilizes the genome. Therefore, Bullerdiek and Rommel (2010) proposed that in this context a plausible assumption is that the cytogenetically unstable cells, displaying sporadic translocations or dicentrics, are those with strong overexpression of the recombinant HMGA2 in a range usually not found during embryonic development.

Depending on further parameters as e.g. the cell type affected, aneuploidy is believed to cause cancer potentially (Boveri, 1902, 1914; Pellman, 2007). However, Weaver et al., 2007 suggested that an increased rate of this common characteristic of cancer cells can be a more effective inhibitor (depending on the level of genomic damage that is induced) than initiator of tumorigenesis (Weaver et al., 2006).

Thus, for biosafety studies cytogenetic analyses are a valuable tool to check the genomic stability of stem cells and their possible malignant transformation. In this field Izadpanah et al. (2008) have described changes during long-term *in vitro*

expansion of adult stem cells of human and rhesus origin. This study has reported that: (1) bone marrow and adipose tissues derived stem cells of human origin had diploid chromosome numbers throughout all passages analyzed; (2) an increasing percentage of tetraploid metaphases was reported for the rhesus bone marrow stem cells and (3) aneuploid cells characterized by random loss of chromosomes are described. As a result it was concluded that these cell populations had lost their ability to maintain chromosome stability during further cell divisions. However, in relation to the genomic instability these results should be interpreted with caution. The reason for concern is based on the consideration that UL are a naturally occurring model of tumor which overexpresses HMGA proteins in subsets of cases. Consequently, tumors with very low expression and those overexpressing either of the HMGA genes are present.

The purpose of the present study is to contribute to the genetic analysis of the UL through investigation of the correlation between cytogenetic changes and the expression of *HMGA* genes. This study has tested this correlation by the following steps. First, a large series of UL was classified karyotypically and (tumor-specific) chromosome aberrations are described. The level of *HMGA2* mRNA in a large series of uterine leiomyomas and *HMGA1* mRNA in subgroup 6p21 with a control group of normal samples was quantified by quantitative RT-PCR. Second, the relationship between the chromosome aberrations and the relative gene expression of *HMGA1* and *HMGA2* was analyzed. Finally, considering the *HMGA2* expression, and the existence of single aberrations and chromosome/chromatid breaks, the correlation between HMGA2 and genome stability was also studied.

2. Materials and Methods

2.1. Tissue samples

The samples of uterine leiomyomas (range of size: 0.5 to 20 cm), analyzed in this thesis (N=309; 161 patients), have been collected from patients (age: 24 to 73 years old) undergoing surgery between May 2006 and December 2009. The samples are provided by the following Bremen Hospitals: Women's Clinic, St. Joseph-Stift Hospital and Department of Obstetrics and Gynecology, DIAKO Evang. Diakonie Hospital. The UL diagnosis has been confirmed by the histopathological analysis in all collected samples. Informed consent to conduct scientific research was obtained from all patients.

In hospital immediately after surgery samples of uterine leiomyomas and myometrium were snap frozen in liquid nitrogen and stored at -80°C. Another part of the tumor (in case of UL) was stored in Hank's solution with antibiotics (200IU/ml penicillin, 200µg/ml streptomycin) for subsequent analysis of cell culture and karyotyping. Fixed, paraffin-embedded tissue sections of the tumor (cut into 5µm sections) have been used for FISH analyses HOPE (Hepes-glutamic acid buffer mediated Organic solvent Protection Effect).

2.2. Cell culture

2.2.1. Cell culture for primary culture

Following a surgery, samples of primary tumors, for the *in vitro* culture of leiomyoma cells, were stored in Hank's solution. The tumor samples for cell culture were minced and treated with 4-6 ml 0.26% (200U/ml) collagenase (Serva, Heidelberg, Germany) and maintained in the incubator with 5% CO_2 at 37°C for 5-8 h. Incubation time varied depending on the texture of the tumors. After centrifugation (1000 rpm for 10 min), the pellet was resuspended and divided in 2 to 4 culture flasks (50 ml). The cells were incubated in 5% CO_2 at 37°C with culture medium (TC 199 with Earle's salts supplemented with 20% fetal bovine serum, 200IU/ml penicillin, 200µg/ml streptomycin). Based on the daily microscope observations of the proliferation rate of primary cultures either medium was changed or cells were trypsinised for subculture.

Regularly, medium was changed two times a week. The first medium change was performed after adhesion of cells in the bottom of flask, at least two days after the primary cell culturing.

2.2.2. Subcultivate for monolayer culture

After a good proliferation rate, cell layer filled a bottom flask, a subcultivate with a 1:2 split ratio was made using trypsin (0.05% Trypsin, 0.02% EDTA in PBS). Floating of cells was controlled by a microscope. The cells were resuspended with 1 ml medium and subsequently divided into two new same size flasks with 5 ml medium and incubated at 37°C and 5% CO_2.

2.3. Cytogenetic methods

2.3.1. Chromosome preparation

For chromosome analysis of exponentially growing cultures of leiomyoma the following method was used. Briefly, to arrest cultured cells during mitosis the cells were incubated in 30 µl colcemid solution (0.06 µg/ml) for 1 hour. Next, the metaphase chromosome spreads were placed in a hypotonic solution (culture medium 20%: aqua dH_2O = 1:6) for 20 minutes and immediately fixated by the solution (methanol: acetic acid = 3:1). After three times washing by fixative and centrifugation the suspension was incubated overnight at 4°C.

2.3.2. Metaphase spread on the objective slides

For metaphases extension the glass slides washed in Ethanol 96% and dH_2O and kept at 4°C were used. Following centrifugation and suction of lotion the suspension was dropped into cold glass slides. Cells prepared on the glass slide were dried overnight at 60°C or at 37°C for 2-3 days.

2.3.3. Staining the chromosomes by G-Banding

The G-band analysis was performed according to the conventional differential staining technique for the identification of chromosomes and their abnormalities. Glass slides were treated (for 7 sec.) in the lotion (after 20 minutes incubation at 37°C of 50 ml banding buffer (41% KH_2PO_4 (1/15 mol) and 59% Na_2HPO_4 x $2H_2O$ (1/15 mol) 15 mg Trypsin powder was dissolved and incubated for 9 more minutes), then transferred immediately to the Giemsa stain (2 ml Giemsa, 5 ml Ethanol 96%, 10 ml Giemsa buffer (50% KH_2PO_4 (1/15 mol) and 50% Na_2HPO_4 x $2H_2O$ (1/15 mol)), and 83 ml dH_2O.) for 10 minutes and finally washed two times by dH_2O before air drying.

2.3.4. Karyotype analysis

Chromosomes have been captured using fluorescence microscope (Axioplan Zeiss, Germany). At least 10 (or more in the myomas with a mosaic karyotype) metaphases were analyzed and 5 of them karyotyped for each case using Macktype 5.5.1 software. The karyotypes were expressed according to the International System for Human Cytogenetic Nomenclature (ISCN, 2005).

2.3.5 Sample selecting for analysis of genomic stability

From the total sample of the UL, based on cytogenetic analysis, two groups of myomas have been selected: first, showing aberrations of chromosomal region 12q14~15 and second, with an apparently normal karyotype. In case of single aberration, only structural aberrations including translocations, deletions, inversions, insertions and ring chromosomes were considered.

Total number of metaphases for each case and the range of karyotyped metaphases for each case were considered. The percentage of metaphases including single aberration or break chromosome/chromatid as well as the percentage of karyotyped metaphases was analyzed for each group. Student's t-test (two sided) was used for the statistical analysis where P values ≤0.05 were considered as significant.

2.4. Molecular methods

2.4.1. RNA isolation

Total RNA was isolated from fresh-frozen tissue samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as following. Tumor tissues (30- 50 mg; in 600 µl RTL buffer (Buffer:Bethamercaptanol = 1 ml:10 µl)) were homogenized using 5 mm bead in a Tissuelyser at 30 Hz for 10 min followed by centrifugation (3 min.) in max speed. RNA isolation was finalized with DNase treatment in accordance with manufacturer's instructions.

Quantification and purity (260/280 nm ratio) of total RNA was determined by spectrophotometery (3 µl RNA with 70 µl RNase free water). The purity of the RNA was within a range 1.8 – 2.0. RNA samples were stored at -80°C prior to RT-PCR analysis.

2.4.2. cDNA synthesis (Reverse Transcription)

Reverse transcription of total 250 ng RNA was carried out with M-MLV reverse transcriptase and random hexamers (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Total RNA was transcribed with a blend of 250 ng DNase double digested RNA in a volume of 10 µl with 1 µl random hexamers (150 ng/µl) and 1 µl dNTP-Mix (10mM). RNA was denatured before transcription at 65°C for 5 min and subsequent cooling on ice for 1 min. After brief centrifugation, 4 µl 5×1st Strand Buffer, 2 µl DTT (0.1M), 1 µl RNase Out and 1 µl M-MLV Reverse-Transcriptase (200 U/ µl) were added, then samples were again centrifugated and incubated in a thermocycler for 10 min at 25°C to allow annealing of the random hexamers. Reverse transcription was performed at 37°C for 50 min (activation of the reverse transcriptase and cDNA synthesis) followed by inactivation of reverse transcriptase (15 min at 70°C).

Controls without enzyme (NoRT) were included for each sample to ensure the absence of DNA contaminations. cDNA was stored at -20°C.

2.4.3. Real-time quantitative RT-PCR

Because of different levels of isolated *HMGA1* mRNA and *HMGA2* mRNA (transcripted to cDNA), a relative quantification method with 18S rRNA as endogenous control was used. 18S rRNA was detected with the following primer/probe set: forward primer: 5'-GGA TCC ATT GGA GGG CAA AGT-3', reverse primer: 5'-AAT ATA CGC TAT TGG AGC TGG AAT TAC-3', probe: TGC CAG CAG CCG C.

Quantitative real-time RT-PCR amplification to detect *HMGA2* was performed on a 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with TaqMan Universal Mastermix and Assay (No. Hs00171569_m1). The *HMGA2*-specific primers in this assay are spanning the boundary between Exons 1 and 2 (forward primer: 5'- GGA CTT GCA CGA CTA A -3', reverse primer: 5'- CCG TAC GTC AAT TGA C -3'). For full-length transcripts of *HMGA1* including HMGA1a and HMGA1b), a set of primers and probe was designed (forward primer: 5'-GGA CCA AAG GGA AGC AAA AA-3', reverse primer: 5'-TTC CTG GAG TTG TGG TGG TTT -3', probe: 6-FAM-AAG GGT GCT GCC AAG ACC CGG-MGB). The probes were diluted in 1:10 concentration for expression of 18S rRNA. Of each cDNA, 2 µl served as template in a final reaction volume of 20 µl. The actual Real- Time PCR was carried out in the following schema:

For *HMGA1* and *18S rRNA*;

Taqman Universal PCR Master Mix	10 µl
Forward- Primer	1.2 µl
Reverse- Primer	1.2 µl
Probe	0.2 µl
H_2O	5.4 µl
cDNA	2.0 µl
Total volume	20 µl

For *HMGA2;*

Taqman Universal PCR Master Mix	10 µl
Assay (Include primers and probe)	1.0 µl
H_2O	7.0 µl
cDNA	2.0 µl
Total volume	20 µl

In order to control and reduce failures, all reactions were run in triplicate. For each run, nontemplate controls (NTC) and reactions without reverse transcriptase (-RT) were included. Reaction condition was as follows: 2 min at 50 °C, 10 min at 95 °C, and 50 cycles of 15 sec at 95 °C and 1 min at 60 °C.

2.5. Fluorescence in situ hybridization

FISH analysis on metaphase preparations was performed after GTG banding of the metaphase spreads.

For determination of rearrangements involving 6p21 and *HMGA1*, respectively, fluorescence in situ hybridization (FISH) was performed on metaphase preparations of the cases with karyotypically 6p rearrangements and myomas with a high expression of *HMGA1* with or without such rearrangements, and also some normal cases as a control.

For FISH, two overlapping clones CTD-2522J1 (GenBank accession numbers AQ280064 and AQ280066) and CTD-2510D13 (GenBank accession number AQ264849 and AQ264850), both located distal to *HMGA1* in 6p21, and two overlapping clones CTD-2524P4 (GenBank accession number AQ310763 and AQ277896) and RP11-140K17 (GenBank accession number AQ385566 and AQ385568), both located proximal to *HMGA1*, in 6p21 were used.

From the results, colocalized signals (green/red) indicate a nonrearranged breakpoint region, whereas separated green and red signals indicate a rearrangement of the

chromosomal region 6p21 and *HMGA1*, respectively. Details were described before (Hashemi Nezhad et al., 2010).

For FISH analysis aimed to finding *HMGA2* rearrangement, three BAC clones were used as break-apart probes. RP11-745O10 (AC078927) and RP11-293H23 (AC012264) are located distal (3‘) to *HMGA2*. RP11-269K4 (AQ478964 and AZ516203) is located proximal (5‘) to *HMGA2*. Labeling was performed by nick translation (Roche Diagnostics, Mannheim, Germany) either with digoxigenin (RP-269K4) or biotin (RP11-745O10 and RP11-293H23). For interphase FISH, formalin-fixed, paraffin-embedded (FFPE) tissue sections were used.

For analysis of the results of interphase FISH, nuclei with two colocalized red/green signals (RG) were scored as normal. Nuclei with one colocalized red/green signal, one single red, and one single green signal (1RG1R1G) were scored as positive for *HMGA2* rearrangement. The details were described before (Klemke et al., 2009).

2.6. Statistical methods (Analysis of gene expression)

The relative expression was calculated by the ΔC_t method, using *18S rRNA* as endogenous control and by calibrating the *HMGA1* and *HMGA2* expression of a myometrial sample matching to a normal myoma. The significance of differential *HMGA1* and *HMGA2* expression between the various groups of myomas (myometrium, myomas with and without 6p21 aberrations in case of *HMGA1* and myomas with and without 12q14~15 aberration in case of *HMGA2*) was determined by the Student’s t-test.

Here, it should be acknowledged that a part of laboratory work was done by Mrs Mahboobeh Tadayyon as her master thesis; the cytogenetic analysis of 56 UL including myomas undergoing long-term cultur, also RNA isolation and PCR of some samples.

3. Results

3.1. Overexpression of *HMGA2* in uterine leiomyomas points to its general role for the pathogenesis of the disease

Klemke et al., 2009

High-mobility group protein A2 was reported to be detected in different benign tumors, as well as some malignancy. The role of HMGA2 protein in the initiation and development of tumors was discussed. Due to the frequency of existence of this protein and high incidence of UL, a large series of myomas (n=180) including tumors with 12q 14~15 rearrangements (n=13), UL with a cytogenetically normal karyotype and matching myometrial tissues (n=51) were analysed by quantitative real-time reverse-transcription polymerase chain reaction.

The highest expression levels for *HMGA2* were observed in UL with rearrangements affecting the chromosomal region 12q14~15 and that was expressed at lower levels in UL without such aberrations. However, the comparison between the expression in myomas and matching myometrial tissues indicated significant (P<0.05) overexpression of *HMGA2* also in the group of fibroids without 12q14~15 rearrangements. This general upregulation of *HMGA2* regardless of the presence or absence of such chromosomal abnormalities suggests a general role of *HMGA2* in the development of the disease.

3.2. Loss of let-7 binding sites resulting from truncations of the 3' untranslated region of HMGA2 mRNA in uterine leiomyomas

Klemke et al., 2010

It was found, that the rearrangements of chromosomal segment 12q14~15, where it encodes the high mobility group At-hook 2 (known as the major subgroup of cytogenetic abnormal UL), leads to an overexpression of *HMGA2*. Therefore, the regulation of this gene is also considerable in the investigation of molecular mechanisms causing an overexpression, especially in those tumors with cytogenetically detectable rearrangements with breakpoints in - or close to 12q14~15

region. MicroRNAs of the *let-7* family are identified as post-transcriptional regulators of *HMGA2*. In a variety of mesenchymal tumors (e.g., leiomyomas, lipomas , and pulmonary chondroid hamartomas) (Ashar et al., 1995; Schoenmakers et al., 1995; Kazmierczac et al., 1996; Wanschura et al., 1996), it is observed that *HMGA2* was targeted by breakpoints situated either intragenically or extragenically 3' or 5' of the gene.

It is presumed that intragenic chromosomal breakpoints might cause truncated *HMGA2* transcripts lacking the part of the 3' UTR, where the let-7 complementary sites (LCS) are located. Therefore this corresponding loss of LCS would stabilize *HMGA2* mRNA by reduction the sensitivity of the transcript against microRNAs of the *let-7* family and finally leads to a higher protein levels in the cells.

So in this study 13 UL with 12q14~15 rearrangements were checked for truncated *HMGA2* transcripts by real-time reverse-transcription polymerase chain reaction. From the total of 13 UL eight leiomyomas with such aberrations, the presence of the complete 3' UTR with all LCS was detected. Five myomas revealed a differential expression of exons 1-2 and 3' UTR with highly reduced 3' untranslated region levels in two of which full-length transcripts were almost undetectable.

Results show that approximately one third of UL, bearing targeted HMGA2 locus affected with chromosomal rearrangements, displayed truncated transcripts. This gives rise to a higher stability of its transcripts and therefore promotes the overexpression of protein. Thus, in most of UL, a loss of let-7 complementary sites is not always responsible for the overexpression of HMGA2; however, it can increase the effects of a transcriptional de-regulation of HMGA2 in a quite small setting of these tumors.

3.3. HMGA2 and the $p19^{Arf}$-TP53-CDKN1A axis: A delicate balance in the growth of uterine leiomyomas

Markowski et al., 2010

Uterine Leiomyomas (UL) are defined to happen through a monoclonal abnormal proliferation of myometrial cells. One of the regular phenomena which exists in premalignant lesions and induces a growth cease especially by two potent growth-

inhibitory pathways as represented by $p16^{Ink4a}$ and $p19^{Arf}$ is the oncogene-induced senescence (OIS). Although there has not been any evidence of relevance between OIS and the development of UL discussed yet, HMGA2 as a major target gene of recurrent chromosomal abnormalities in UL has been related directly with the repression of the *Ink4a/Arf* (*CDKN2A*) locus. Taking this point into consideration, this study investigated the probable contribution of HMGA2 to the development of leiomyomas through repressing this locus. The result was contradictory. It was observed that UL typically exhibits higher levels of $p19^{Arf}$ mRNA comparing to myometrium, and likewise there could be seen a higher level of UL with 12q14~15 rearrangements rather than UL with other cytogenetic aberrations. Moreover, it was found that the existence of a significant correlation between the expressions of $p19^{Arf}$ and *CDKN1A* activates senescence rather than apoptosis in UL. Notably, the size of tumors was also correlated with the levels of *HMGA2*, $p19^{Arf}$, and *CDKN1A.* This shows that the $p19^{Arf}$ pathway counteracts with enhancing the growth potential. It can be presumed that the UL probably performs a program already present in their original cell, and when activated, protects the genome, for which the enhanced proliferation can be named as an example. The research concluded that, based on the results, the $p19^{Arf}$-TP53-CDKN1A pathway plays an important role in controlling the growth and genomic stability of uterine fibroids.

3.4. 6p21 rearrangements in uterine leiomyomas targeting *HMGA1*

Hashemi Nezhad et al., 2010

HMGA2 as targeting gene in the most frequent cytogenetic abnormal subgroup of UL is discussed in the initiation and development of myomas in different studies. *HMGA1* is another member of high-mobility group protein A which is targeted in another non-random aberrant subgroup of UL with 6p21 rearrangements. An overexpression of *HMGA1* was detected before resulting from such rearrangements in the locus of the gene in the short arm of chromosome 6 (Sornberger et al., 1999; Tallini et al., 2000). However, it seems that yet no study quantifying the expression of HMGA1 mRNA in UL of this subtype has been performed. Thus, aimed to quantify the expression of *HMGA1* mRNA in UL, the expression of *HMGA1* was analyzed in a series including tumors with 6p chromosomal aberrations (n=7) and cytogenetically normal tumors

(n=8) as a control group by quantitative real-time reverse-transcription polymerase chain reaction. The average expression level in the 6p21 group was found to be 5.6 times higher than that in the control group and with one exception all cases with 6p21 alteration revealed a high expression of *HMGA1* mRNA than cytogenetically normal tumors. However, what distinguishes the tumors of both types is the level of overexpression of *HMGA* genes compared to myometrium. Nevertheless, the increase of the average expression of *HMGA1* mRNA in these cases was significantly less strong than that of *HMGA2* mRNA in case of 12q14~15 aberrations identified in the previous studies.

3.5. 12q14~15 aberrations do not enhance the chromosomal instability in uterine fibroids

Hashemi Nezhad et al., submitted

Resuming the previous study of high-mobility group protein A and their roles in the development of tumors, this time genome stability in the UL and its correlation with HMGA2 expression was considered and investigated as a possible role for HMGA2 protein. For this aim, three groups of myomas have been selected based on cytogenetic analysis. The first one showed the rearrangements of chromosomal region 12q14~15 (with a high expression of *HMGA2)* without single aberration or chromosome/chromatid break (N=16); the second one, an apparently normal karyotype without single aberration or chromosome/chromatid break (N=151) as the cells with low levels of *HMGA2* considered as the control group; and the third, all UL affected with single aberration or chromosome/chromatid break (N=46) as cases with higher genome instability. In case of single aberration only structural aberrations including translocations, deletions, inversions, insertions, and ring chromosomes were considered.

Total number of metaphases for each case and the range of karyotyped metaphases for each case were considered. The percentage of metaphases including single aberration or break chromosome/chromatid as well as the percentage of karyotyped metaphases was analysed for each group.

From a total of 46 affected UL, 31 samples showed only one impression of single aberration or break and 15 UL showed more than one (2-4). In most analyzed

samples, the affected metaphases included one chrb/chtb or single aberration. However, in 5 myomas two alterations occurred in the same metaphase. In 4 of these 5 UL two chtb or chrb were observed together. And just in one case (Myoma 659.2, with 4 different occurrence including translocation, two different ring chromosomes and chrb), r(13) and chrb(15) took place together in the same metaphase (Fig. 3.9). In comparison between all UL with 12q14~15 rearrangements and all normal myomas there was no significant difference between the percentage of impression with a single structural aberration or chromosome/chromatid break.

Concerning the gene expression, there was no significant difference between UL without single aberration or chrb/chtb and targeted UL with single aberration or chrb/chtb. Likewise, the expression of *HMGA2* was analyzed between affected UL with just one event and myomas including more than one chrb/chtb or single aberration as more instable cells. Results confirm that there was no difference between these two groups of affected UL.

In order to investigate the role of *HMGA2* in the stability of genome, these two groups of myoma cells (normal group and 12q14~15 group) were compared with malignant cells, as well with higher level of HMGA2 in comparison with normal cells. Concerning the genome stability, contrary to normal cells that have good and high stability, the malignant cells are known to have higher genome instability.

If HMGA2 would be considered as a positive factor in induction of genome instability, it is expected that malignant cells show a far higher expression of *HMGA2* than benign and stem cells, and likewise, these cells should have a high instability in their genome. However it is proved that malignant cells express the *HMGA2* in a lower level than benign tumors including 12q14~15 rearrangements. It is true that in stem cells, this group has a high expression of *HMGA2* despite their high genome stability too.

It is unlikely that the stem cell chromatin associated protein HMGA2 which can be found plentifully during embryonic life (Rogalla et al., 1996; Li et al., 2007), impairs the integrity and stability of genome. Especially in the phase of the embryonic life, the strong proliferative activity should happen simultaneous with a proper supply of genetic integrity. Therefore, it seems plausible to assume that the cells having 12q14~15 abnormalities make a protection for genome in spite of their high HMGA2. This could be due to the existence of other factors which are associated with this

high HMGA2 that counteract the effect of HMGA2 in the induction of genome instability, e.g. high expression of p^{19Arf}.

3.6. Unpublished results

3.6.1. Cytogenetic analysis

In this study, 261 UL from 141 patients have been investigated by cellular and molecular analysis. The age of patients ranged between 24-80 years old, where age group between 40 and 50 was the largest (Fig. 3.1).

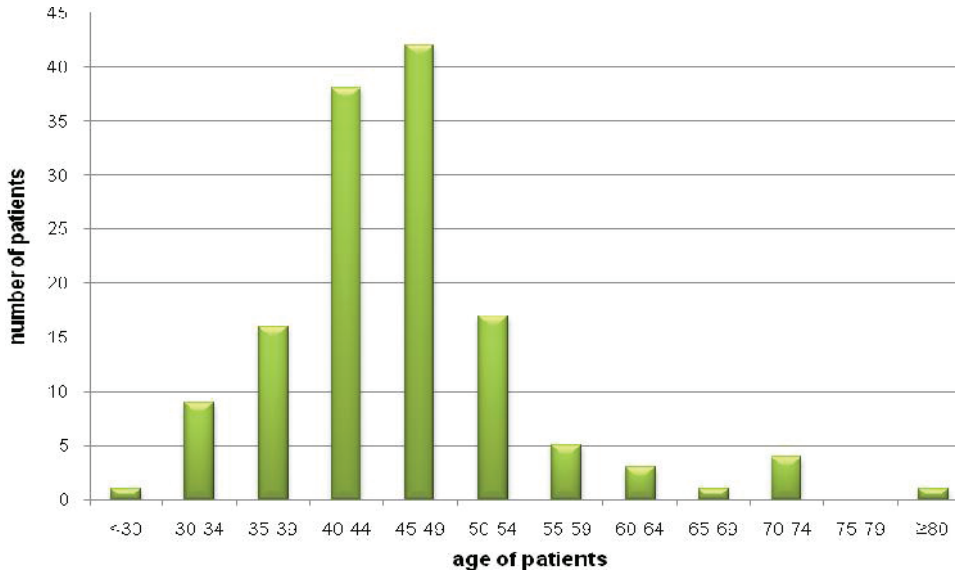


Fig. 3.1: Distribution of age in the patients with uterine leiomyomas.

The size of tumor was in a range of 0.5–20 cm (Fig.3.2). Aberrant UL showed never a size smaller than 1 cm (Fig. 3.4).

Based on the cytogenetic karyotyping, UL were subdivided into two major groups. The first group includes 76 UL that showed chromosomal aberrations and the second one, 185 UL with an apparently normal karyotype. 65 patients had at least one tumor with an aberrant karyotype. The frequency of abnormal karyotypes was 29.1% per nodule and 46.1% per patients. UL with an abnormal karyotype were further analysed and subdivided into four major cytogenetic subgroups (12q14~15 rearrangements, del(7q22), 6p21 rearrangements, and trisomy 12) (Fig. 3.3).

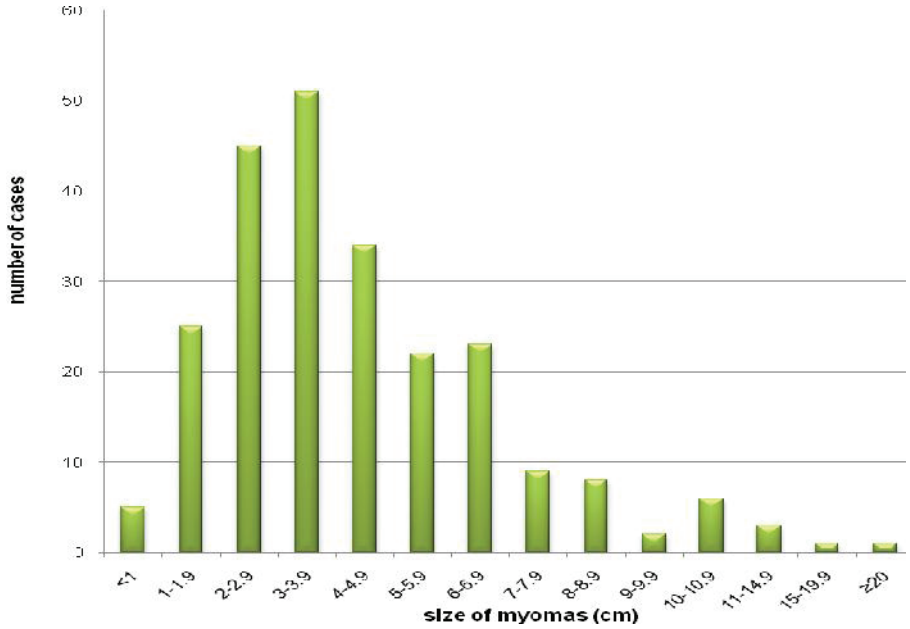


Fig. 3.2: Distribution of the size of tumors in the uterine leiomyomas.

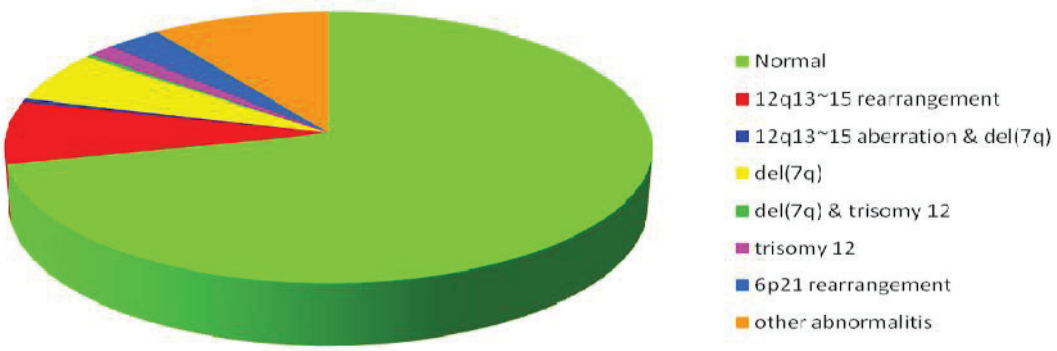


Fig. 3.3: Frequency of major cytogenetic subgroups in uterine leiomyomas.

Aberrant UL were also analyzed for mosaic (aberrant/normal, aberrant/aberrant) or non-mosaic karyotype (Tab. 3.1). From total of 76 UL with an aberrant karyotype, 46% showed a non-mosaic karyotype and 54% revealed a mosaic karyotype (56.1% A/N, 24.4% A/A, 12.2% A/A/N, 2.4% A/A/A, and 4.9% with a cp karyotype).

Tab. 3.1: Karyotype and clinical data of all aberrant UL. Karyotypes belong to primary material or first chromosome analysis and include also the structural single aberrations and FISH karyotype for some samples (UL with a high gene expression despite normal karyotype and *vice versa*).

No	Case No.	Lab No.	Size (cm)	Age (y)	Karyotype	Non-mosaic or mosaic: A/N or A/A
1	501	3	10	48	46,XX[36]. nuc ish(269K4,745O10/293H23)x2(269K4 sep 745O10/293H23x1)[23/100]	N.M
2	503	6	4	40	46,XX,inv(5)(q15q31~33),t(12;14)(q15;q24)[13]	N.M
3	513	15	5	42	46,XX,del(7)(q22q31)[3]	N.M
4	523.1	25A	-	33	45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),-22[16]/44,XX,-9, t(12;14),der(14)t(12;14),der(19)t(9;19),-22[1]	N.M
5	523.2	25B	-	33	45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),-22[15]. Ish t(12;14)(269K4+,745O10/293H23-;269K4+,745O10/293H23+), der(14)t(12;14)(269K4+,745O10/293H23+)[11/11]	N.M
6	524	26	-	40	46,XX,del(7)(q22q34) or (q11.2q31)[2]	N.M
7	526	32	3	42	45,XX,rob(13;14)(q10;q10)c[15]	N.M
8	532.2	34B	5	39	46,XX,inv(1)(p13q32)[16]	N.M
9	532.3	34C	-	39	46,XX,inv(1)(p13q32)[14]	N.M
10	533	35	6	41	46,XX,r(1),t(1;12;14)(p36.3;q14;q24)[19]	N.M
11	535.1	37A	5	43	47,XX,+10[2]/46,XX[9]	M(A/N)
12	535.2	37B	4	43	46,XX,t(8;11)(p23;q13.1)[6]/47,XX,+12[2]/46,XX[15]	M(A/A/N)
13	535.5	37E	3	43	46,XX,del(7)(q11.2?)[2]/46,XX[12]	M(A/N)
14	536.1	38A	6	46	46,XX,del(7)(q22q31.2)[13]	N.M
15	536.3	38C	3	46	46,XX,del(7)(q21.2q31.2)[6]	N.M
16	538.3	40C	6	36	46,XX,ins(2;3)(q32;q22q27)[3]	N.M
17	541	43	7	37	46,XX,t(12;14)(q15;q24)[5]/46,XX[9]	M(A/N)
18	542.1	44A	7	46	46,XX,inv(7)(p21q22)[3]/46,XX[5]	M(A/N)
19	544.2	46B	4	49	46,XX,del(7)(q22q32)[2]/46,XX[4]	M(A/N)
20	545	47	5	47	46,XX,t(12;14)(q15;q24)[9]/46,XX[3]	M(A/N)
21	546	48	6	45	47,XX,+12[9]/46,XX[3],46,XX,del(7)[1]	M(A/N)
22	547.1	49A	2.5	73	46,XX,der(12),der(14)?ins(14;12)[8]/46,idem,r(1)[4]	M(A/A)
23	549.4	51D	6	49	48,XX,+der(6),-8,+11,+mar[11]	N.M
24	550	53	-	30	46,XX,add(1)(p),der(1)t(1;3?)(q;q),der(3)del(3)(p) or add(3)(p)t(1;3)(q;q),der(4)t(3;4)(p;q)[17]	N.M
25	551.2	52B	5	45	48,XX,+4,+12[11]/48,XX,+4,del(7)(q22q31.2),+12[8]/48,XX,t(1;3), +4,+12[1]/48,XX,t(2;8),+4,del(7),+12[1]/47,XX,del(7)+12[1]	M(A/A)
26	552.2	54B	10	49	46,XX,t(2;12)(q33;q13)[17]. ish t(2;12)(745O10/293H23+;269K4-) [4/4]	N.M
27	554.2	56B	2.0	41	46,XX,del(13)(q13or14q31)[7]/46,XX[9]/47,XX,+12[1]	M(A/N)
28	556	57	5	42	46,XX,t(3;5;12)(q23~25;p13~15;q13~15)[11]/45,XX,t(3;5;12) (q23~25;p13~15;q13~15),-22[10]	M(A/A)

No	Case No.	Lab No.	Size (cm)	Age (y)	Karyotype	Non-mosaic or mosaic: A/N or A/A
29	559.2	61B	10	73	46,XX,der(7)del(7)(q11.2q31)inv(7)(q11.2q36)[16]/46,XX[2]	M(A/N)
30	564.2	67B	3	65	46,XX,del(7)(q21.2q31.2)[12]/46,XX,t(1;3)(q25;q26),del(7)(q21.2q31.2)[4]	M(A/A)
31	573	76	5	37	46,X,t(X;2)(p11.4;p25),del(7)(q11.2q22),inv(9)(p11q13)c[10]/46,XX,inv(9)(p11q13)c[11]/46,XX,del(7)(q11.2q22),inv(9)(p11q13)c[1]	M(A/A)
32	576.5	79E	6	49	46,XX,del(7)(q22q32),r(16)(pterqter)[5]/46,XX[8]/46,XX,del(7),+12,-16[1]	M(A/N)
33	579	82	1.5	49	46,XX,t(12;15;14)(q15;q26;q24)[20]/46,XX[1]	N.M
34	580	83	8	40	46,XX,der(7)del(7)(p)del(7)(q),t(12;14)(q15;q24)[3]/46,XX,idem,der(8)add(8)(q),der(10)add(10)(q)[16],45,XX,del(7)(q32),t(12;14),-19 [1]	M(A/A)
35	584	87	-	63	46,XX,t(6;14)(p23;q24)[6]/46,XX,t(6;14)(p23;q24),tas(14;21)(pter;qter)[11]/46,XX[2]/47,XX,+12[1]	M(A/A/N)
36	591.2	94B	2.5	47	46,XX,del(7)(q22q32)[9]/46,XX[1]	N.M
37	593	96	1	44	47,XX,+12[3]/46,XX[15]	M(A/N)
38	595.1	98A	3	50	46,XX,del(7)(q22q32)[3]/46,XX[5]/47,XX,+X[1]	M(A/N)
39	596	99	12	49	46,XX,ins(2;12)(q34 or q35;q24.3 or q24.1q13),inv(4)(q27q31.3)[22]	N.M
40	597.2	100B	-	37	46,XX,del(7)(q11.2q21)[15]	N.M
41	601	104	5	40	45,XX,del(1)(p3?),-16[4]/46,XX[11]	M(A/N)
42	603.2	106B	3	35	46,XX,del(7)(q?)[8]	N.M
43	607	110	4	44	44,XX,der(1)t(1;?),der(3),der(5)t(5;?),-6,der(11)?t(11;15)(q25;q22),del(15)(q22),der(15)t(15;?),-19[25]. ish der(6)?t(6;?)(CTD-2524P4+,RP11-140K17+),der(?)?t(6;?)(CTD-2522J1+,CTD-2510D13+)[13/13]	N.M
44	608	111	5	46	46,XX,ins(8;1)(q12;p22p13)[22]	N.M
45	609	112	4	33	46,XX,der(3)t(3?;6)(p23;q?),der(6)(q),der(8)(p)[13]/46,XX[4]	M(A/N)
46	610.3	113C	3.5	53	46,XX,t(6;10)(p23;q23)[5]/46,XX[7]	M(A/N)
47	612	115	6	44	46,XX,der(1)r(1;?),t(12;14)(q15;q24)[4]/46,XX,t(12;14)(q15;q24)[13]/45,XX,r(1),t(12;14),der(15)t(15;18),-18[1]/42,XX,der(1)r(1;?),-4,dic(11;?;15),t(12;14)(q15;q24),-15,-15,-20[1]. ish t(12;14)(269K4+,745O10/293H23-;269K4+,745O10/293H23-)[10/10]	M(A/A)
48	613.4	117D	4.5	39	46,XX,t(6;11)(p23;q21)[4]/46,XX[12]	M(A/N)
49	614.1	116A	2	56	46,XX,del(7)(q22q32)[2]/46,XX[21]/46,XX,t(1;1)[1]/46,XX,der(1)[1]	M(A/N)
50	615.1	118A	5	47	46,XX,del(6)(q15 or q16)[19]	N.M
51	616	119	8	48	45,XX,-22[17]	N.M
52	617	120	8	44	46,XX,der(1)del(1)(p22),der(3)?t(1;3)(p22;q?),der(5)del(5),der(12)t(12;?)(q24.3;?),-14,-20,+mar1+mar2[6]	N.M
53	618.2	121B	3	38	42~46,X,-X[6],-1[19],t(1;8)(p22;q24)[11],der(1)[6],del(3)[3],add(6)[19],-8[6],der(8)[4],t(9;14)[19],-10[6],-11[6],-13[6],-14[19],-22[15],+mar1[18],+mar2[18],+mar3[6],+mar[6][cp19]/46,XX[1]. ish t(6;?)(CTD-2524P4+,RP11-140K17+;CTD-2522J1+,CTD-2510D13+)[10/10]	CP

No	Case No.	Lab No.	Size (cm)	Age (y)	Karyotype	Non-mosaic or mosaic: A/N or A/A
54	621.1	125A	2.5	42	46,XX,t(6;11)(p21;p15)[7]/46,XX[14]. ish t(6;11)(CTD-2524P4+,RP11-140K17+;CTD-2522J1+,CTD-2510D13+)[5/17]	M(A/N)
55	622	126	1.5	48	46,XX,del(10)(q24 or q25)[11]/46,XX[5]	M(A/N)
56	624.1	128A	3	42	46,XX,del(3)(q22~q23)[19]	N.M
57	624.2	128B	2.5	42	46,XX,del(3)(q25q27)[15]	N.M
58	625.2	129B	6	46	41~46,XX,-1[16],-2[16],dup(7)(q34q11.2)[16],-13[16],-16[6],+r1[10],+r2[5],+mar1[3],+mar2[2],+mar3[3][cp16]	CP
59	626.1	130A	6	52	46,XX,der(3)t(3;?)(p21;?),del(13)(q12q14),der(16),der(19)t(3;19)(p21;q13.4)[16]/46,XX[1]	N.M
60	628.1	132A	4	57	46,XX,t(2;4)(q33;q25)[14]/46,XX[1]/46,XX,t(2;4),t(7;9)[1]	N.M
61	628.2	132B	1.5	57	46,XX,?ins(12;14)(q15;q31q24)[5]/46,XX[14]/46,XX,?ins(12;14)(q15;q31q24),der(4)t(4;14)(q;q10),-14,+mar[1]	M(A/N)
62	630	134	8	44	46,XX,der(2)del(2)(p)del(2)(q),der(11)t(2;11;?)(q;p;?)[13]/46,XX,idem,del(8),-17,+mar[6]/46,XX[3]	M(A/A/N)
63	632	136	4	47	46,XX,t(12;14)(q15;q24)[12]/46,XX,del(4)(q31orq32),der(10)?t(10;14)(q24;q32),t(12;14)(q15;q24)[9]/45,XX,der(1)?t(1;2),-2,add(7)(?q36),t(12;14)(q15;q24)[2]	M(A/A/A)
64	635	139	-	59	46,XX,der(10),del(12)(q13 or q14)[18]	N.M
65	641.1	149A	6	41	46,XX,r(1),t(5;8)(q35;q21)[6]/46,XX[2]/45,XX,-1,t(5;8)[2]/45,XX,r(1),der(5)t(5;8),-8[1]/44,XX,r(1),-3,t(5;8),-21[1]/44,XX,r(1),-3,t(5;8),-14[1]/41,X,-X,r(1),der(5)t(5;8),-8,-11,-13,-21[1]/45,XX,-21[1]	M(A/A/N)
66	643.2	151B	6	52	46,XX,t(12;14)(q15;q24)[14]. ish 6p21(CTD-2524P4,RP11-140K17,CTD-2522J1,CTD-2510D13)x2[9/9]	N.M
67	645	153	8	46	45,XX,r(1),der(13;14)(q10;q10)t(12;14)(q15;q24)[20]/44,XX,-1,der(13;14)(q10;q10)t(12;14)(q15;q24)[6]/44,XX,der(13;14)t(12;14),-20[1]	M(A/A)
68	646	154	9.5	47	46,XX,t(2;12)(p21;p13)[11]. ish 6p21(CTD-2524P4,RP11-140K17,CTD-2522J1,CTD-2510D13)x2[17/17]	N.M
69	652	160	1	71	46,XX,del(7)(q21q31)[4]/46,X,t(X;2)(p22.3;q31),del(7)(q21q31)[2]/46,XX[8]/47,XX,+8[1]	M(A/A/N)
70	654.2	162B	2.5	43	47,XX,+12[4]/46,XX,[12]	M(A/N)
71	656.1	164A	1.1	73	46,XX,add(6)(q?13),der(6)t(6;8)(q?13;q13),der(?8)r(?8)[8]/46,XX[6]	M(A/N)
72	656.2	164B	2	73	46,XX,add(6)(q?13),der(6)t(6;8)(q?13;q13),der(?8)r(?8)[6]/45,XX,add(6)(q?13),der(6)t(6;8)(q?13;q13),der(?8)r(?8),dic(9;19)(q34;p13)[2]/45,X,-X,add(6),der(6),der(?8)[2]/45,XX,add(6),der(6),der(?8),-21[1]/44,XX,add(6),der(6),-7,der(?8),-18[1]	M(A/A)
73	656.3	164C	2.5	73	46,XX,add(6)(q?13),der(6)t(6;8)(q?13;q13),der(?8)r(?8)[11]/46,XX[1]/45,XX,add(6),der(6),der(8),12[1]/45,XX,add(6),der(6),der(8),-18[1]/45,XX,add(6),der(6),der(8),-15[1]/45,XX,add(6),der(6),der(8),-8,-20[1]/44,XX,-6,der(6),-8[1]/45,XX,dic(2;7),add(6),der(6),der(8)[1]/44,XX,dic(2;9),add(6),der(6),-8[1]	N.M

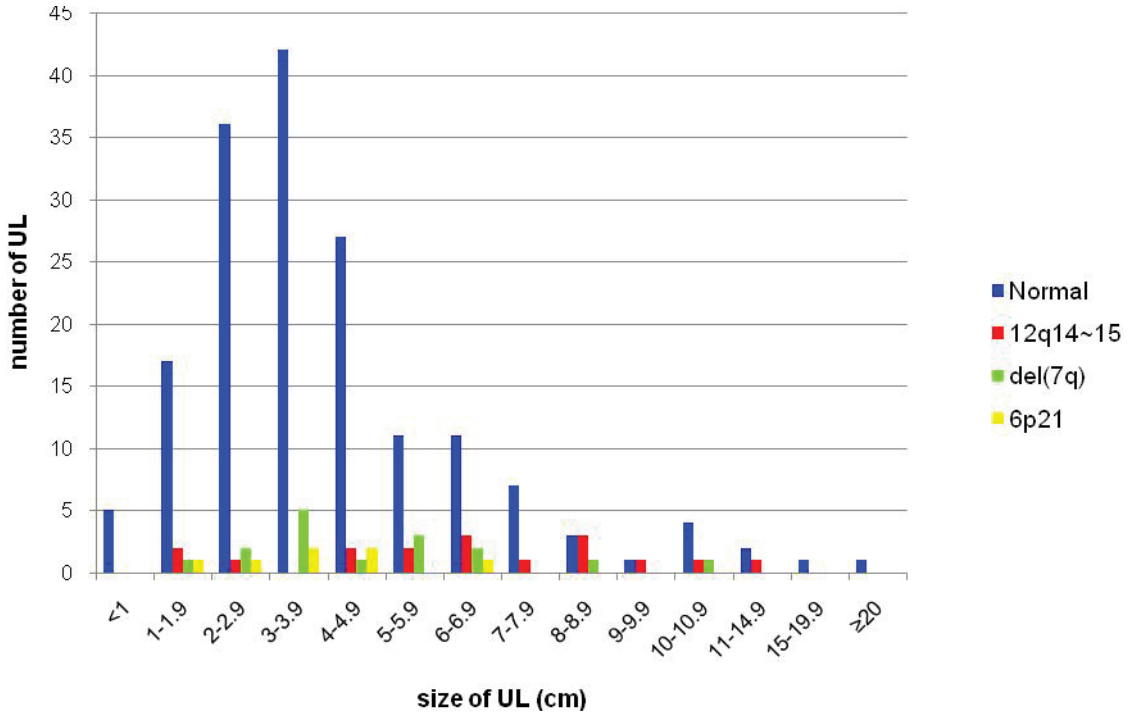
No	Case No.	Lab No.	Size (cm)	Age (y)	Karyotype	Non-mosaic or mosaic: A/N or A/A
74	656.4	164D	6.5	73	46,XX,add(6)(q?13),der(6)t(6;8)(q?13;q13),der(?8)r(?8)[12]/45,XX, idem,-10[1]/45,XX,idem,-19[1]/45,XX,idem,-8,-17,-18,-20[1]/43,X,-X, idem,-13,-21[1]	N.M
75	656.6	164F	8	73	46,XX,add(6)(q?13),der(6)t(6;8)(q?13;q13),der(?8)r(?8)[2]/45,XX, add(6)(q?13),der(6)t(6;8)(q?13;q13),-8[11]/44,XX,add(6),der(6), der(?8),-10,-18[1]/44,XX,add(6),der(6),-8,-22[1]/44,XX,add(6),der(6), -8,-20[1]	M(A/A)
76	658.1	166A	1	47	46,XX,t(6;10)(p21;q22)[13]/46,XX[8]/44,XX,t(6;10)(p21;q22),-14,-20 [1]/44,XX,-7,-20[1]. ish t(6;10)(CTD-2524P4+,RP11-140K17+;CTD2522J1+,CTD-2510D13+)[7/19]	M(A/N)

Abbreviations: M: Mosaic, N.M: Non-Mosaic, A/N: Aberrant/Normal, A/A: Aberrant/Aberrant, -: unknown.

Tumor size was analyzed in different cytogenetic subgroups of UL aiming to investigate significant differences between these groups, as well as, finding the correlation between the size of tumors and their chromosomal changes. The correlation between size of tumor and their chromosomal changes in four major cytogenetic subgroups of UL (normal, 12q14~15 rearrangements, 6p21 rearrangements, and del(7q)) was analyzed. As reported before (Hennig et al., 1999), UL with 12q14~15 changes were significantly larger in myomas rather than those belonging to the other groups. However there was no significant difference (P = 0.585) between normal and 6p21 UL. As it is described before, the mosaic karyotype myomas (normal/aberrant) are significantly smaller in size than those solely composed of abnormal cells (Rein et al., 1998) (Fig. 3.4).

Most available studies refer to t(12;14) or involvement of 12q14~15 region as most prevalent subgroup in cytogenetically abnormal UL (for example Gordon et al., 2003; Ligon and Morton, 2001). However Sreekantaiah et al. (1994) and Pandis et al. (1991) reported that a partly deletion of the long arm of chromosome 7 was the most common aberration in UL. The present study also confirmed that 12q14~15 rearrangements were most frequent aberration (27.6%) followed by del(7q) (23.7%) in myoma (Tab. 3.2).

A



B

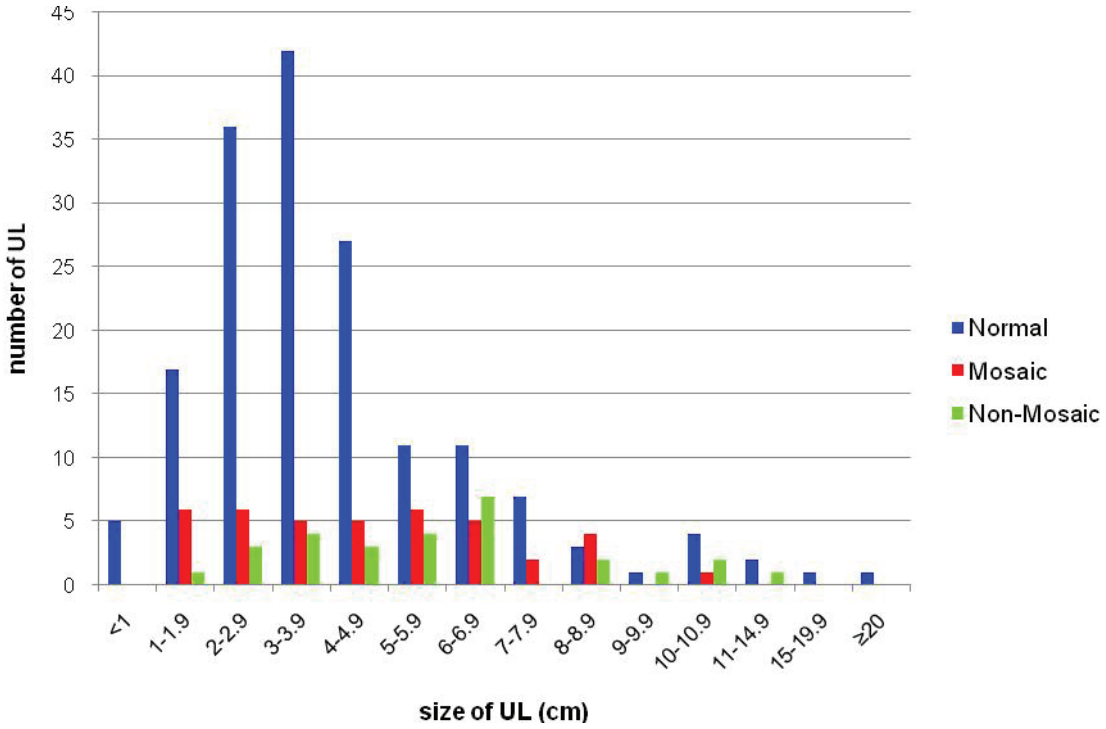


Fig. 3.4: A and B. Analysis of tumor size in different cytogenetic subgroups of UL.

All rearrangements of chromosome region 14q24 were with 12q14~15 changes (14 UL). However rearrangements of 12q14~15 without simultaneous involvement of 14q23-24 have been reported in seven leiomyomas (Tab. 3.1).

Tab. 3.2: Frequency of different aberration in aberrant leiomyomas.

Chromosomal rearrangement	**Number of cases**	**Percentage of different aberration in total aberrant cases (76UL)**
12q14~15 rearrangement	21	27.6%
del(7q)	18	23.7%
Chr.14 aberration	15	19.7%
14q24 rearrangement	14	18.4%
Chr.1 aberration	16	21.1%
1p rearrangement	10	13.2%
6p21 rearrangement	8	10.5%
3q rearrangement	7	9.2%
Trisomy 12	5	6.6%
10q rearrangement	5	6.6%
Monosomy 22	4	5.3%
Chr.5 aberration	4	5.3%
Chr.X aberration	3	3.9%
complex karyotype	2	2.6%
Ring chromosome	12	15.8%

From total 76 aberrant UL in the present study, 18 myomas revealed a karyotype with loss of chromosomal band 7q22 of which seven cases showed the del (7q) as the sole change. In eight UL this anomaly was present in a mosaic state accompanied with normal cells, however in three of them other structural aberrations were observed with del(7). The t(12;14) that is accompanied by del(7q) was present just in one myoma, the same range for trisomy 12 and a ring chromosome (ring 16). Interestingly, a translocation of chromosomes X and 2 was observed in two UL with mosaic karyotype formula of del(7q) and normal cells that are the whole cases including structural rearrangements of chromosome X in total analyzed UL.

Trisomy of chromosome 12 as one type of non random abnormality in UL was always observed in mosaic form in the way that an extra chromosome 12 was revealed in lesser number of cells (Tab. 3.1).

Another often discussed anomaly in UL, monosomy of chromosome 22, was observed in five cases. In three of those cases, loss of a chromosome 22 was accompanied by rearrangement of 12q14~15. One case showed just monosomy 22 and one case had a composite karyotype (Tab. 3.1). In myoma with complex karyotype formula (myoma 618.2) with different structural and numerical abnormalities, most cells (15/19 total analyzed cells) revealed loss of one chromosome 22.

In the present study, from total 76 aberrant UL, 12 (15.8%) myomas showed a ring chromosome. Five of them had a ring (1) of which four UL showed ring (1) with a t(12;14). The last one was accompanied with a t(5;8). Five UL revealed a karyotype with a r(?8) and involvement of chromosomes 6 and 8 with t(6;8). Notable is that all of these UL were removed from one patient (myoma 656). One myoma (576.5) had a r(16) that was accompanied with del(7q). Last case (myoma 625.2) with ring chromosome revealed a complex karyotype with duplication of chromosome 7 and monosomy of chromosomes 1, 2, 13, and 16 in all cells and two ring chromosomes as marked chromosomes without any rearrangement in chromosomes 12 and 14. Present findings showed r(1) as the secondary change after translocation or insertion of chromosomes 12 and 14 in two tumors. Accompanied by this change in one myoma, in other cases ring chromosomes were detected in mosaic karyotype with a normal clone and/or with a clone of cells losing ring one.

Notably, the chromosomes 17 and 18 were never found abnormal (Tab. 3.1).

3.6.2. Molecular analysis

In undertaking molecular analysis, in addition to 12q 14~15 group the expression of *HMGA2* was also analyzed in the cases with trisomy of chromosome 12 (N=5). Analysis of the expression of *HMGA2* showed no significant difference (*P*=0.268) between the group of UL with trisomy 12 and normal group (Fig. 3.5). There was also a significant difference between cells with trisomy of chromosome 12 and 12q 14~15 group.

The expression of *HMGA1* mRNA expression was also checked in two cases of 12q 14~15 rearrangements. The average of relative *HMGA1* was 29.8 fold in comparison with normal group (7.2 fold) and 6p21 group (45 fold) (Fig. 3.6).

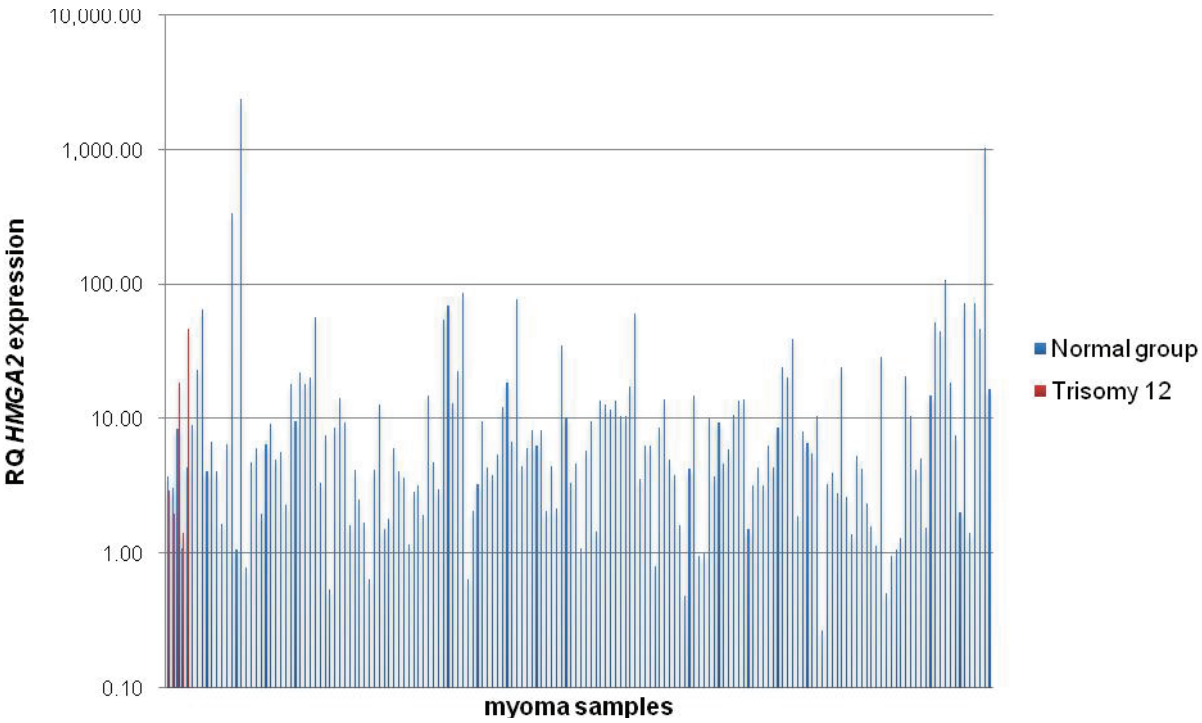


Fig. 3.5: The comparison of *HMGA2* expression between normal group of UL and myomas with trisomy 12.

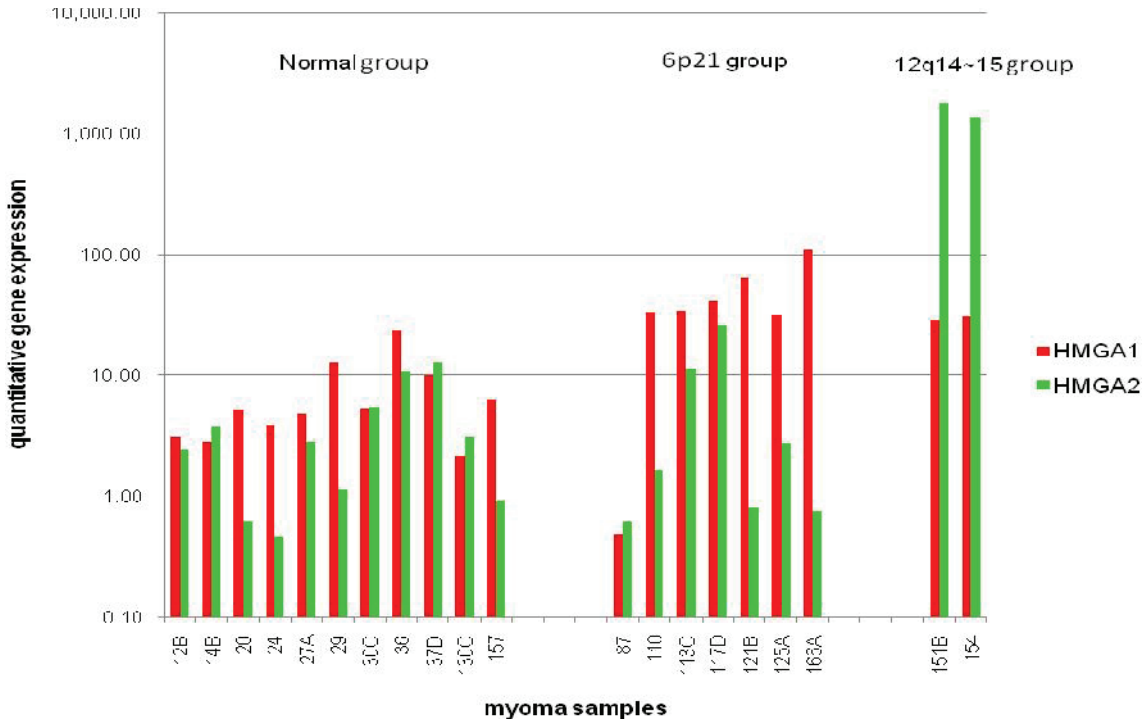


Fig. 3.6: Quantitative *HMGA1* and *HMGA2* expression in myomas with normal karyotype, 6p21 rearrangements, and 12q14~15 rearrangements.

3.6.3. Long–term *in vitro* expansion of myoma cells

Recent papers have linked HMGA proteins with an increased genomic instability due to a reduced capacity to repair DNA damage. Thus, in order to do a biosafety study for more investigation of the roles of HMGA proteins, cytogenetic analyses were used as a valuable tool to check the genomic stability of stem cells. For this aim, four UL (one myoma with 12q14~15 rearrangement and high expression of *HMGA2*, one case with 6p21 rearrangement and overexpression of *HMGA1*, and in the same time of culturing each aberrant case, one normal sample as control) were investigated for the cytogenetic changes during long-term in vitro expansion.

Chromosome analysis of myoma 646 showed a rearrangement of p arm of chromosome 12 (46,XX,t(2;12)(p21;p13)). At the same time the molecular study of *HMGA2* in a quantitative real time RT-PCR test resulted in a high expression of *HMGA2*. Thus, a FISH analysis with *HMGA2* probes was performed for this tumor sample. Metaphase FISH revealed a cryptic insertion of q15 of chromosome 12 in p13 of that chromosome which leads to a rearrangement of the location of *HMGA2* gene on the long arm of chromosome 12. Consequently this case was investigated as a myoma with 12q14~15 rearrangements in long-term culture for 140 days until the 22nd passage. The changes in chromosomes 2 and 12 stayed stable in all passages. No more changes were observed in the later subcultures until the 11th passage. In the last analyzed passage (P. 22), different clones of cells were observed. First t(2;12) was seen in all clones of cells and also the only change in one clone. Other clones including secondary changes had a ring chromosome 9, just one cell showed a der(9) instead of r(9). Third clone of cells in addition to these two changes revealed a karyotype with 45 chromosomes because of monosomy of chromosome 6. A near-tetraploid karyotype with 92 chromosomes was observed in a few cells starting from P.11. Changes of chromosome 9 started from P.12 by existence of der(9), monosomy of chromosome 6 happened in P.18 and later, and ring chromosome 9 was observed in P.22(Fig. 3.7).

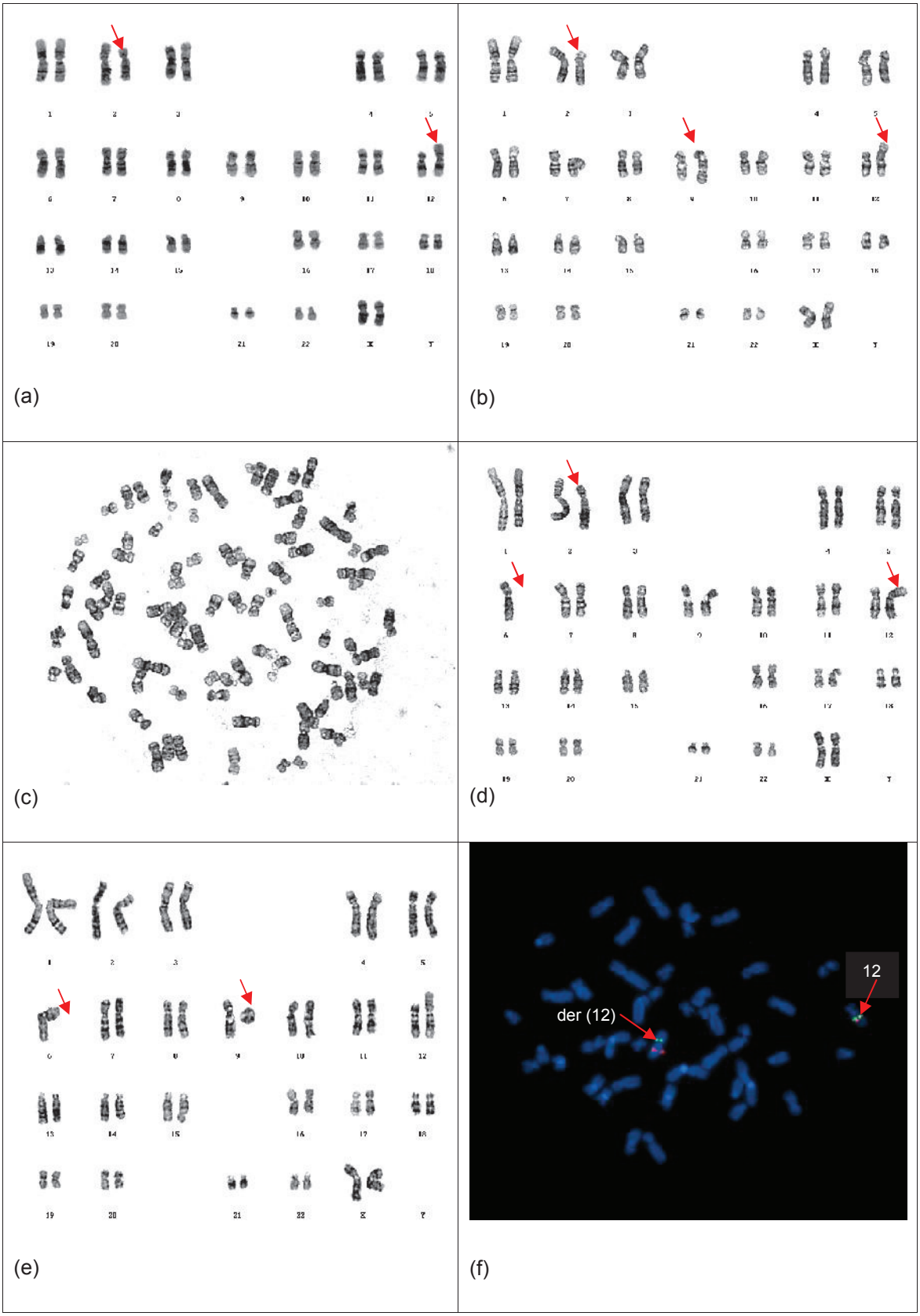


Fig. 3.7: Karyograms of different passages (P) and the metaphase FISH figure of myoma 646 with 12q14~15 rearrangement in long-term culture.

(a): Primary material; 46,XX,t(2;12)(p21;p13)[11]; (b): P.12; 46,XX,t(2;12)(p21;p13),der(9); (c): P.11; Tetraploidy; (d): P.18; 45,XX,t(2;12)(p21;p13),-6; (e): P.22; 46,XX,t(2;12)(p21;p13),-6,r(9);

(f): Primary material, metaphase dual-color FISH indicating a rearrangement of the *HMGA2* locus. The green fluorescent probe (RP11-269K4) located proximal (5‘) to *HMGA2*, the red fluorescent probes (RP11-745O10 and RP11-293H23) located distal (3‘) to *HMGA2,* colocalized signals (green/red) indicate a nonrearranged breakpoint region, whereas separated green and red signals indicate a rearrangement of the chromosomal region 12q14~15 and *HMGA2*, respectively.

At the same time, the myoma 641.2 with an apparently normal karyotype and *HMGA2* expression in range of normal UL was cultured as the control for the aberrant case. The cells were grown up to P.14 during 117 days. From the results, no chromosomal changes were observed in the whole passages, just a few tetraploid metaphases were revealed in the middle passages but not in the first and the last of them. They were in a range of 2-4 metaphases in each objective slide.

Myoma 658.1 with karyotype formula: 46,XX,t(6;10)(p21;q22)[13]/46,XX,[8] and a very high expression of *HMGA1* was considered as a case with rearrangement of 6p21 and cultured for 147 days until 25th passage. This mosaic pattern was observed only in the first passage followed by the growth of only normal clone of cells. No other cytogenetic changes were observed in the long-term culture of these cells with a high *HMGA1* expression (Fig.3.8). Just tetraploid metaphases were observed from P. 5 and in higher rate in P. 8. These cells showed a reduced rate in last passages.

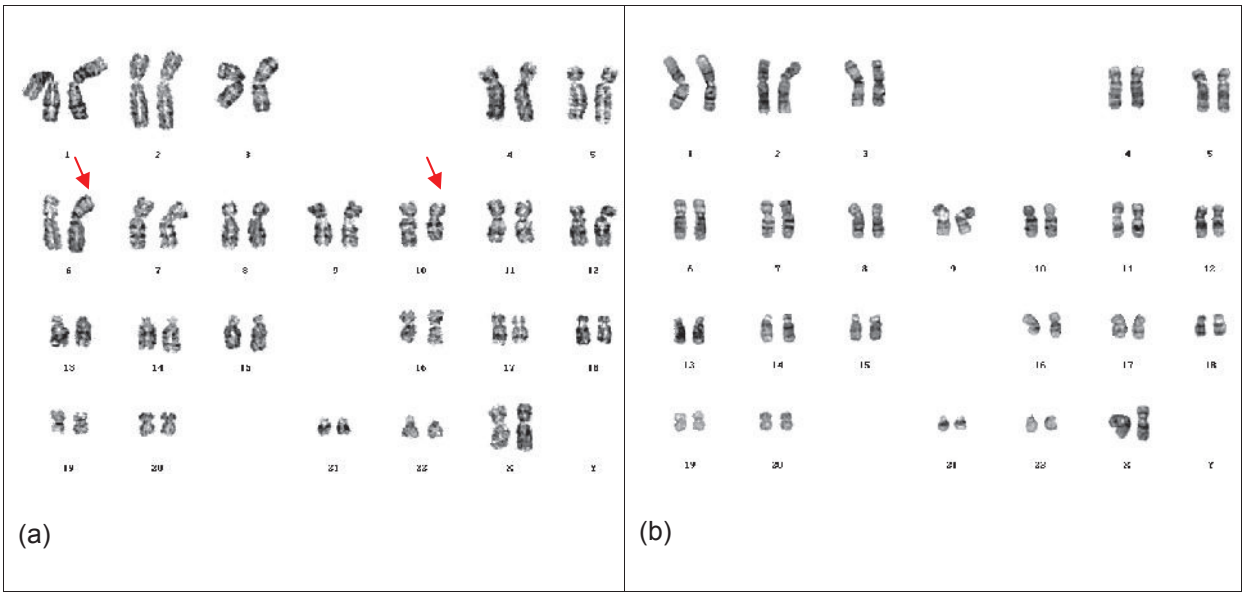


Fig. 3.8: Karyograms of primary material and last passage (P) of myoma 658.1 with 6p21 rearrangements.

(a): Primary material; 46,XX,t(6;10)(p21;q22)[13]/46,XX[8]; (b): P.25; 46,XX

As a control for myoma 658.1, the chromosome analysis of the normal case (myoma 659.2) in long-term culture was also carried out. The cells of this case were cultured for 82 days and until 15th passage. The cultured cells of primary material of the normal case showed a normal karyotype but with different single cell aberration including translocation and ring chromosomes. All passages of this case showed a normal karyotype (Fig.3.9). This normal UL did not reveal any tetraploid metaphase either.

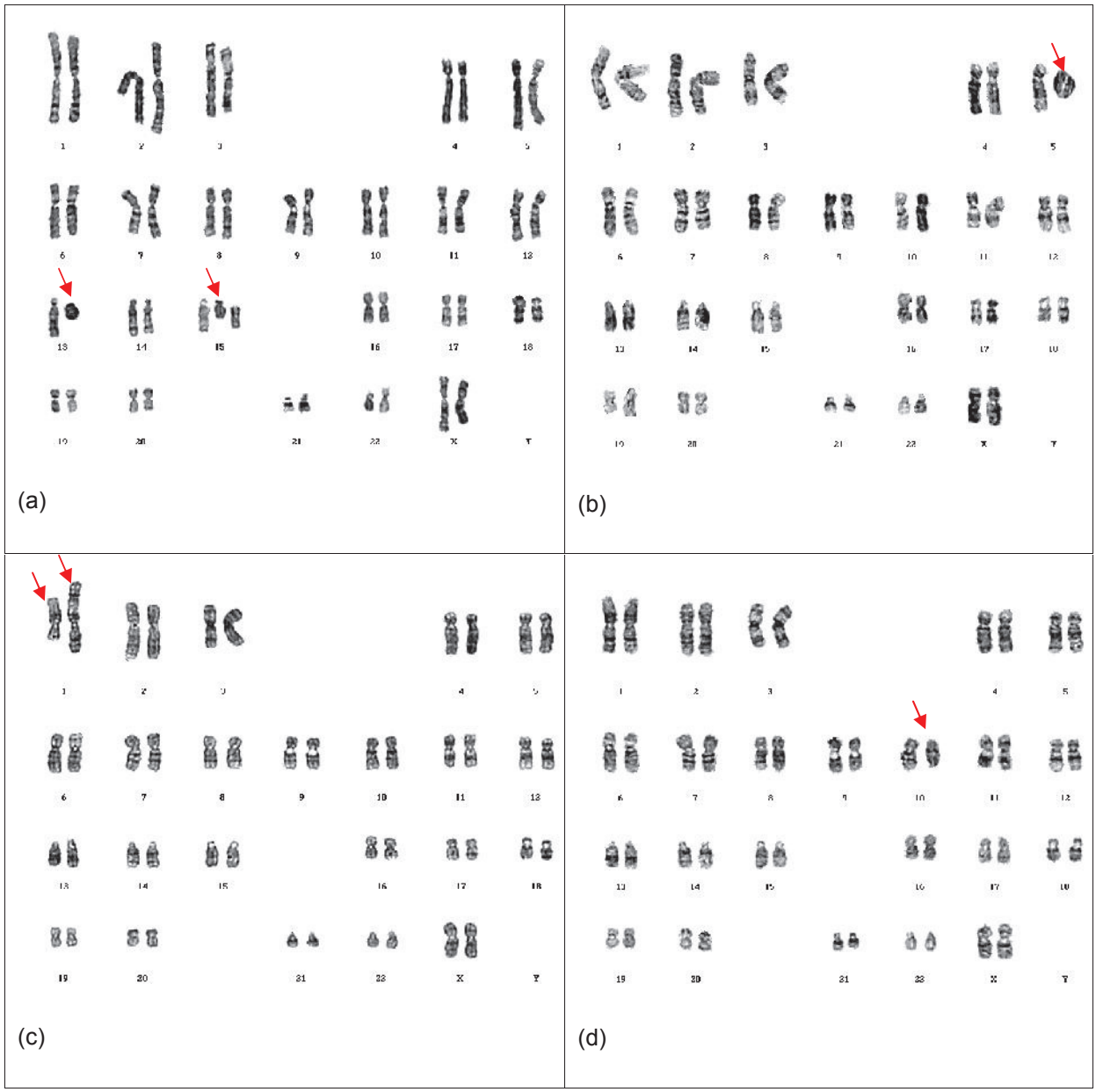


Fig. 3.9: Karyograms of myoma 659.2 with a normal karyotype and different single aberrations in the primary culture.
(a): 46,XX,r(13),chrb(15)(q); (b): 46,XX,r(5); (c): 46,XX,t(1;1)(p36;q21); (d): 46,XX,?r(10)

4. Discussion

UL are by far the most common gynecological tumors occurring in about 70-80% of all women in their reproductive age (Cramer and Patel, 1990; Day Baird et al., 2003a; Heinemann et al., 2003), and are accounted as the major reason for hysterectomy in the United States, including approximately one-third of all hysterectomies (Wilcox et al. 1994). Despite their high prevalence, concerning their rare malignant transformation (<0.1% (Sandberg, 2005)), the etiology and pathogenesis of these benign muscle tumors remain poorly understood.

The present study aimed at a better understanding of the biopathology of leiomyomas, investigating a large series of UL in the cytogenetic and molecular genetic analysis. This study tries to find the correlation between chromosomal changes and gene expression of two involved genes in two major cytogenetic subgroups of UL, those two genes which code the proteins of high mobility group AT-hook proteins. This relation was analyzed to understand in more detail thes role of HMGA proteins in the growth of mesenchymal benign tumors and their role in the genome instability. Considering the abundant expression of these stem cell chromatin associated proteins in embryo in the high proliferative stage with high stability of genome, revelation of their function in the tumors could help us find more about their biopathology and malignant transformation.

The available studies indicate that between 25-50% of diagnosed UL show chromosomal aberrations (see for example; Sandberg, 2005; Gross et al., 2003; Flake et al., 2003; Morton, 2000; Hennig, 1999). In this context only one study from Japan stands alone (Kataokaa et al., 2003). The Japanese study based on the analysis of a large series of myoma found abnormal karyotype only in 7% of UL (Tab. 4.1). The substantially different results of the Kataokaa et al., (2003) study and its comparability to other studies may partially be explained by a biased selection of the investigated tumors (e.g. a selection based on the size) and factors related to race. To mitigate the selection bias and reduce the error, the present study is based on UL samples obtained after hysterectomy or myomectomy and analysis of all detected myomas (following a standard protocol). Our results of the cytogenetic analysis of 261 myomas indicate that 29.1% of myomas have an abnormal karyotype, which in fact confirms the findings of the previous studies.

Tab 4.1: Cytogenetic analysis of uterine leiomyomas in various studies.

Total Samples	Normal Karyotype	Aberrant Karyotype	Region of study	reference
261	185 (70.9%)	76 (29.1%)	Germany	Present study
166	145 (93%)	21 (7%)	Japan	Kataokaa et al., 2003
197	147 (75%)	50 (25%)	Germany	Hennig, 1999
182	129 (71%)	53 (29%)	Belgium	Brosens et al., 1998
114	73 (64%)	41 (36%)	USA	Rein et al., 1998
76	41 (54%)	35 (46%)	Arizona	Meloni et al., 1992
13	6 (46%)	7 (54%)	Massachusetts	Rein et al., 1991
63	39 (62%)	24 (38%)	Sweden	Pandis et al., 1991
90	59 (65.5%)	31 (34.5%)	Sweden	Pandis et al., 1991
35	21 (60%)	14 (40%)	Pennsylvania (U.S)	Kiechle-Schwarz et al., 1991
40	27 (67.5%)	13 (32.5%)	Italy	Vanni et al., 1991
189	145 (76.7%)	44 (23.3%)	Swedwn	Nilbert et al., 1990
9	5 (55.5%)	4 (44.5%)	Arizona.	Fan et al., 1990

Discussing the role of the cytogenetic abnormalities in the etiology of uterine fibroids posed a question on whether these chromosomal changes are the first or second event in the UL. Addressing this question, some studies do agree with the hypothesis confirming the secondary nature.

The secondary nature of the chromosomal rearrangements is supported by the findings of the studies suggesting that mosaic karyotype (normal/aberrant) myomas are of the monoclonal origin (Mashal et al., 1994); and they are significantly smaller in size than those solely composed of abnormal cells (Rein et al., 1998). Moreover, it should be noted that at least half of UL have a normal karyotype.

It is proposed that UL mostly occur in the reproductive age (Kane, 2002). In this study the age of patients ranged between 24-80 years old where age group between

40 and 50 was the largest, with only few cases in patients under 30. The age indicated above is the age of a patient at the time of operation, and not the incidence of myoma or the age of their diagnosis. Sometimes, depending on the symptoms and complications of tumor, the time period between incidence and diagnosis, as well as diagnosis and treatment takes a very long time.

A regular size of 0.5 to 20 cm was commonly observed. The largest group in this series of removed UL was 3-4 cm. It has been argued that the loss of genetic material from chromosome 7 results in a less optimal growth in a myoma (Xing et al., 1997). This finding was also supported by another study that found the deletion of 7 subgroup is involved in most of mosaic tumors while they are actually smaller in size rather than chromosomally normal myomas (although the difference was not statistically significant) (Morton, 1998). Rein et al. (1998) described that mosaic karyotype myomas (normal/aberrant) are significantly smaller in size than those solely composed of abnormal cells. Later, Hennig et al. (1999) showed that myomas with 12q14~15 changes are significantly larger in size than myomas with a normal karyotype and the tumors with deletion of chromosome 7 are significantly smaller in size. Based on the results of the present study, mosaic UL had smaller size than non-mosaic aberrant myomas but this difference was not significant (P=0.070). Analysis of the tumor size in different cytogenetic subgroups revealed an agreement with Hennig et al. (1999) (P=0.008). However there was no significant difference (P=0.456) between normal myomas and UL with a deletion of 7q. Moreover no significant difference (P=0.574) was noted between normal group and UL with 6p21 rearrangements.

Our analysis of multiple myomas indicates that even in a uterus with a multiple myoma often just one nodule shows abnormal karyotype. The present finding was also earlier confirmed in the studies by Pandis et al. (1994) and Kataokaa et al. (2003).

4.1. Molecular cytogenetic analysis

Most available studies refer to t(12;14) or involvement of 12q14~15 region as most prevalent subgroup in cytogenetically abnormal UL (for example Ligon and Morton, 2001; Gordon et al., 2003), with a frequency of about 20% of aberrant myomas (Nilbert and Heim, 1990; Meloni et al., 1992; Gordon et al., 2003). Within this context only one study from Germany showed a higher frequency (Hennig, 1999). This study,

based on the analysis of a large series of myoma, found rearrangements of 12q14~15 in 46% of cytogenetic aberrant UL. However Sreekantaiah et al. (1994) and Pandis et al. (1991) reported that a deletion of part of the long arm of chromosome 7 was the most common aberration in UL. The present study also confirmed that 12q14~15 rearrangements were most frequent aberration (27.6%) followed by del(7q) (23.7%) in myoma.

This chromosomal abnormality of chromosome 12 is of particular interest because the same region of 12q is also commonly rearranged in a variety of other mesenchymal solid tumors e.g. lipomas, breast fibroadenomas, endometrial polyps, pulmonary chondroid hamartomas (PCH), hemangiopericytomas, angiomyxomas (Turc-Carel et al., 1986; Bullerdiek et al., 1997; Calabrese et al., 1991; Mandahl et al., 1993; Vanni et al., 1993; Ozisik et al., 1994; Dal Cin et al., 1995; Fletcher et al., 1995). As it is identified, the cytogenetic subtypes are correlated properly with a different molecular pathogenesis of the disease. The critical gene located in the chromosome 12q14~q15 region is *HMGA2* (Ashar et al., 1995; Schoenmakers et al., 1995). In normal cells, transcripts of the *HMGA2* gene code primarily for the full-length HMGA2 protein, a member of high mobility group protein AT-hook of which the expression has been detected in UL with 12q14~15 rearrangements, but not in matched normal myometrium (Gattas et al., 1999; Klemke et al., 2009). Quade et al. (2003) mentioned that in the 12q15 rearrangements, mostly breakpoints are situated 5‘ and a few at the 3‘ location (usually found outside the *HMGA2* coding region). It was later supported by Klemke et al. (2009) that an extragenic breakpoint upstream but in closer proximity of *HMGA2* can be sufficient to trigger its overexpression. Despite a wide distribution of breakpoints, intragenic as well as extragenic (Kazmierczak et al., 1995; Hennig et al., 1996; Schoenmakers et al., 1999; Kurose et al., 2000; Mine et al., 2001; Takahashi et al., 2001; Quade et al., 2003), the molecular alterations resulting from the cytogenetic deviations generally seem to include an upregulation of the gene (Tallini et al., 2000; Gross et al., 2003; Klemke et al., 2009) and primarily affect the expression rather than the protein sequence (Quade et al., 2003). Thus, overexpression of the full-length transcript or a truncated or chimeric protein HMGA2 seems to be sufficient to trigger tumorigenesis (Klemke et al., 2009). The term overexpression refers to an expression exceeding the matching myometrium. It is found that high HMGA2 protein levels are a factor correlated with a worse progression of malignant neoplasias, and *HMGA2*

rearrangements can be frequently seen in benign tumors of mesenchymal origin (Tallini and Dal Cin, 1999; Fedele et al., 2001). Moreover, it is observed that HMGA2 can act as a biomarker for some types of malignant tumors (Mahajan et al., 2010). HMGA2 expression could be of prognostic significance in some cancers, e.g. non-small cell lung cancer (Wu et al., 2008), metastatic breast cancer (Langelotz et al., 2003), retinoblastoma (Mu et al., 2010), and cell carcinomas of the oral cavity (Miyazawa et al., 2004). It can also serve as a valuable detector for distinguishing malignant tumors from benign ones (Belge et al., 2008; Lappinga et al., 2010).

In description of the role of HMGA proteins in tumorigenesis, Bullerdiek (1997) promoted the hypothesis that the overexpression of HMGA2 can induce an embryonic chromatin configuration in cells and then empowers them with a stem-cell like behavior. Further studies concerning the HMGA2 expression in embryonic stem (ES) cells also supported this assumption (Li et al., 2006, 2007; Pfannkuche et al., 2009). Li and colleagues (2006, 2007) confirmed that HMGA2 is consistently associated with inter- and metaphase human ES cells chromatin and admited it as a regulator of key developmental genes in these embryonic cells. Pfannkuche et al.(2009) later came up with the idea that, by means of three independent DNA binding domains, HMGA2 essentially promotes the plasticity of ES cell chromatin and is associated with the maintenance of an undifferentiated cell state.

Although it was shown that UL with normal karyotypes can have cryptic inversions of 12q (Wanschura et al., 1997; Weremowicz and Morton, 1999), nevertheless, at least 50% of UL show a normal karyotype, meaning that no cytogenetically visible chromosomal rearrangements can be seen and, moreover also by molecular cytogenetic methods there is no evidence for the existence of submicroscopic alterations of the *HMGA2* locus in a considerable number of these cases (Weremowicz and Morton, 1999). In addition, Klemke et al. (2009) indicated an overexpression of HMGA2 mRNA also in karyotypically normal tumors. This study by using quantitative RT-PCR in a large series of UL revealed a higher *HMGA2* expression in cytogenetically normal karyotype fibroids in comparison to their matched myometrium. This outcome suggests a more general role of HMGA2 and its overexpression in the development of UL, and not only in the subgroup characterized by 12q14~15 alterations.

The fact that the overexpression of HMGA2 is even present in the UL without observed cytogenetically 12q14~15 rearrangements elevates the hypothesis that one

of the main factors in the genesis of UL is a high level of a stem-cell chromatin associated protein. For clarifying the *HMGA2* overexpression despite a normal karyotype detected by usual cytogenetic techniques; it could be said that, besides a lack of cryptic *HMGA2* rearrangement, distinguished by classical cytogenetics, a selection of a group of cells without translocation during cell culture may admit the apparent normality of karyotype.

The variability in the basic level of HMGA protein among the samples of the same cytogenetic group of tumors might be explained by “mosaicism”. If this mutation was an early or later event during tumor development, then the greater or lesser proportion of cells would express the gene (Williams et al., 1997). Sometimes taking a part of a big mosaic tumor could lead to a selecting of different clones of the same tumor for different analysis. Another possibility can be the reflection of a specific type of mutation that has occurred in each tumor (Williams et al., 1997), or reflecting alterations during the menstrual cycle. These kind of alterations in the patterns of gene expression have been investigated before, e.g., by Kayisli et al. (2007).

In spite of the monoclonal origin of UL (Townsend et al., 1970; Mashal et al., 1994; Hashimoto et al., 1995; Zhang et al., 2006), the mutations of the gene are not necessarily responsible for the overexpression of HMGA2. In several recent studies, it is observed that microRNAs of the let-7 family regulate HMGA2 post-transcriptionally (Lee and Dutta, 2007; Mayr et al., 2007; Park et al., 2007; Shell et al., 2007; Kumar et al., 2008; Motoyama et al., 2008; Peng et al., 2008). Nevertheless, the molecular mechanism which induce an overexpression, especially where cytogenetically translocations are detectable with breakpoints in or close to the chromosomal location domain of 12q14~15, are still to be studied and identified. Likewise, there are also reports on the reduced expression of let-7 family members in UL (Peng et al., 2008). Although the down-regulation of miRNAs decreases HMGA2 expression, other factors like loss of the let-7 complementary sites (LCS) in the 3’ UTR of HMGA2 can also be responsible for the deficiency in let-7- mediated regulation (Mayr et al., 2007). The study by Klemke et al. 2010 shows that although the loss of let-7 complementary sites is not associated with the HMGA2 overexpression in most UL, it seems to raise the effects of a HMGA2 transcriptional deregulation in a small subset of UL (Klemke et al., 2010).

The 14q23~q24 region, which is most often fused to 12q14~15, is also involved in several mesenchymal benign tumors, including uterine leiomyomas, pulmonary chondroid hamartomas, and endometrial polyps (Walter et al., 1989; Nilbert and Heim, 1990; Rein et al., 1991; Dal Cin et al., 1993, 1995; Fletcher et al., 1995; Vanni et al., 1995; Kazmierczak et al., 1995, 1996). Two candidate genes in this region of chromosome 14 are *ESR2* (Estrogen Receptor Beta Gene) (Pedeutour et al., 1998) and *RAD51L1* (a member of the *RAD51* recombination repair gene family) (Schoenmakers et al., 1999). The *RAD51L1* gene is introduced as a translocation partner for *HMGA2* in leiomyoma (Schoenmakers et al, 1999; Ingraham et al., 1999). This gene is involved actively in DNA repairs recombination, although such a recombination is not detected to be catalyzed by the RAD51L1 protein (Takahashi et al., 2001) and may be essential for cell proliferation (Shu et al., 1999).
Some other partner genes for *HMGA2* have also been described, i.e., the *COX6C* gene at 8q22~q23 (Kurose et al., 2000), the *ALDH2* gene at 12q24.1 (Kazmierczak et al., 1995), the enhancer of invasion (*HEI10*) gene at 14q11 (Mine et al., 2001).

Another cytogenetic subgroup is characterized by aberrations of 6p21, the location of another member of *HMGA* family, *HMGA1* (Friedmann et al., 1993). Rearrangements of band 6p21 and overexpression of HMGA1 respectively have been observed frequently in various mesenchymal tumors, including lipomas (Tallini et al., 1997), pulmonary chondroid hamartomas (Xiao et al., 1997), breast hamartomas (Dal Cin et al., 1997), and uterine leiomyomas (Sornberger et al., 1999; Tallini et al., 2000). Moreover, results of the present study proved the higher expression of *HMGA1* in the 6p21 group than that in the normal group (Hashemi Nezhad et al., 2010). Notably, compared to fibroids with a normal karyotype, in these cases the upregulation of the HMGA1 mRNA was much less stronger than that of HMGA2 mRNA in the case of 12q14~15 aberrations. As showed in different studies, HMGA1 plays a role in both activation and suppression of the transcription of several genes, therefore, over expression of HMGA1 in UL might facilitate activation or repression of a variety of genes relevant to tumor growth and biology (Williams et al., 1997).
Furthermore, neoplastic transformation was associated with HMGA1 expression in different human neoplasias such as prostatic cancers (Tamimi et al., 1993; 1996), thyroid neoplasias (Chiappetta et al., 1995; 1998), pancreatic duct cell carcinomas (Abe et al, 2000), breast carcinmas (Chiappetta et al., 2004), hepatocellular

carcinomas (Chang et al., 2005), lung cancers (Sarhadi et al., 2006), and leukaemias.(Pierantoni et al., 2003). Furthermore, differential expression and prognostic value of HMGA1 was shown in the subtypes of some tumors (van der Zee et al., 2010).

Further studies have observed that the role of HMGA1a protein in both virus integration and viral genome expression in host cells of immunodeficiency virus type 1 (HIV-1), human papilloma virus type 18 (HPV-18) (Hindmarsh et al., 1999; Farnet and Bushman, 1997; Bouallaga et al., 2003; Henderson et al., 2004) and all of the herpes simplex virus type 1 (HSV-1) (Matta and Panagiotidis, 2008).

In addition to the 6p21 group and normal cases, *HMGA1* expression was analysed also in two UL with 12q14~15 aberrations (myoma 151B and myoma 154). Interestingly, the level of HMGA1 mRNA in these myomas (average=29.8) were also higher than normal group (average=7.2), and much closer to the range of 6p21 group (average=45) than that in normal UL (Fig. 3.6). As Williams et al. (1997) suggested, these tumors may have acquired small mutations, undetectable by standard cytogenetic techniques that lead to the ectopic expression of HMGA1 in the absence of cytogenetic abnormalities. Aimed to reduce such missing, FISH analysis was performed by using *HMGA1* probes. From the results, no split was revealed for *HMGA1* gene in these two myomas. It is mentioned that, despite the apparent differences in interacting partners of both genes (Arlotta et al., 1997), they have a great extent of sequence and structural similarity (Tallini and Dal Cin, 1999) and a high homology in their DNA-interacting domains. Therefore, it can be suposed that HMGA1 and HMGA2 are able to replace each other functionally, at least in part.

Findings of this study do also agree with Williams et al. (1997) concerning the lack of a significant correlation between HMGA1 levels and tumor size (Fig. 3.4A).

del(7)(q22), another non-random cytogenetic abnormality in UL is present in some studies as most frequent abnormality in myomas (Nilbert and Heim, 1990; Pandis et al., 1991; Meloni et al., 1992) and by the others as the second common rearrangement in this tumor (Rein et al., 1991; Vanni et al., 1991; Ligon and Morton, 2000). del(7)(q22q32) is present in about 17-24% of karyotypically abnormal UL (Nilbert and Heim, 1990; Hennig, 1999; Ligon and Morton, 2000). It is shown that probably del(7) coexists with t(12;14) or t(1;6) (Nilbert et al., 1989). This idea suggests that del(7q) is involved with the karyoypic evolution of leiomyoma, although

the t(12;14) often occurs as the sole abnormality (Sait et al., 1989). Sargent et al. (1994) found that these deletions and rearrangements of 7q22 region are more consistent in UL than in any other tumors. It was shown that UL with chromosome 7 deletions or translocations are usually present in the mosaic form accompanied with normal cells (Xing et al., 1997). From present results, in eight UL this anomaly was present in a mosaic state accompanied with normal cells, however in three of them other structural aberrations were observed with del(7). Although based on the finding of this anomaly as the sole alteration in some UL, Ligon and Morton (2000), proposed a possible role as an early genetic event for it in UL.

The observations of cell culture showed a slow proliferation pattern of myoma cells *in vitro* in tumors with a deletion of chromosome 7, especially in the cases showing this rearrangement as the sole change and subsequently a difficult chromosome analysis with low number of reached metaphases. This suggests a possibility of the same changes in the missed samples of chromosome analysis. This suggestion means a higher frequency for this subgroup of aberrant UL. It was before stated that cells with the del(7)(q22q32) are more likely to persist in cultures when the t(12;14) is also present (Sandberg, 2005).

A large number of genes or growth factors, particularly those localized in the commonly deleted area of 7q22 have been identified. A possible relationship between insulin resistance genes and rearrangements at 7q21.3 has been postulated (Sell et al., 1998). Existence of a novel tumor suppressor gene for uterine smooth muscle tumors on the chromosome region 7q22 was suggested by the relatively high frequency of loss of heterozygosity (LOH) in this region (van der Heijden et al., 1998). It was indicated that approximately 30 genes have been mapped to the 7q22 region, from which the plausible candidate for smooth muscle tumor suppressors is the *COL1A2* gene, which encodes the a-2 chain of collagen type 1. Considering the role of *COL4A6* gene in the hereditary syndrome diffuse leiomyomatosis, *COL1A2* is an available candidate (Hudson et al. 1993). Nevertheless, *COL1A2* seems to map proximal to the upper boundary of the minimal deletion unit. Sandberg (2005) believed that events at 7q may not be crucial to leiomyoma development. While it is detected before that in the leiomyomas with del(7q) as the sole abnormality, *HMGA2* expression was not found, its expression was confirmed in a tumor with t(12;14)(q15;q24) and a del(7q) and another with a t(2;3;12)(q35;q21;q14) as the sole change (Henning et al., 1997).

Analysis of the expression of *HMGA2* in the group of trisomy 12 as one type of non random abnormality in UL showed no significant difference (*P*=0.268) between the group of UL with trisomy 12 and normal group (Fig. 3.5). It could support the hypothesis that rearrangements by intragenic as well as extragenic breakpoints of *HMGA2* gene can result in an overexpression (Quade et al., 2003; Klemke et al., 2009).

Another often discussed anomaly in UL, monosomy of chromosome 22, was observed in five cases. Loss of one chromosome 22 has been previously detected in myomas (Gibas et al., 1988; Turc-Carl et al., 1988; Nilbert et al., 1989; Pandis et al., 1990, 1991). Pandis et al. (1991) concluded that monosomy 22 is probably a non-random secondary abnormality. The present finding is not contrary to this idea; however one case showed monosomy 22 as the sole abnormality.

In the present study, 12 (15.8%) aberrant myomas showed a ring chromosome. Five of them had a ring (1) of which four UL showed ring (1) with a t(12;14). Ring formation and structural rearrangements of chromosome 1 as the secondary changes in UL with t(12;14)(q14~15;q23~24) were discussed before by Nilbert et al. (1988). It is likely that two mechanisms are involved in producing ring chromosomes. The most common one is the breaking of both arms of the chromosome and loss of distal segments. The loss of chromosomal material happens as the result of the subsequent fusion of the ends. This classical pattern of ring chromosome formation commonly induces multiple phenotypic effects (Gardner and Sutherland, 1989). Another type of ring chromosome is created by telomere-to telomere fusion. In this case little or no loss of chromatin may happen, and if the ring chromosome would be lost, it might result in significant consequences, and then monosomy. It is assumed that the instability of ring chromosome is produced by sister chromatid exchange within the ring which may generate unstable variants (Therman, 1986).
Sawyer et al. (1992) illustrated that a primary cytogenetic event in solid tumors can be the telomeric association which is a mechanism able to induce chromosome instability through generating subclones with unstable chromosome intermediates, and therefore ring chromosome formation and consequently monosomy.
In a recent review by Gebhart (2008) that listed cytogenetically analysed leiomyomas with ring chromosomes, it is reported that in most of the cases the non-random

involvement of chromosome 1 in the rings can be detected by cytogenetic and molecular cytogenetic techniques. Polito et al. (1999) who applied FISH with PAC-clones for *HMGN2* (high-mobility group nucleosomal binding domain 2, located in 1p36.1), could not find any signal on the r(1): This can mean that *HMG17* does not have any mechanistic role in leiomyoma which is similar to what is seen in other high-mobility proteins. In a few analyzed uterine leiomyomas, r(1) or r(1;?) were found particularly as the sole karyotypic anomaly which is a part of a 2n=46 karyotype (Nilbert and Heim, 1990; Kiechle-Schwarz et al., 1991; Polito et al., 1999). This is the same as Sandberg's (2005) suggest, maintaining that, rings containing chromosome 1 may be secondary changes in these tumors; while Pandis et al. in 1991 had suggested that r(1) formation is a preferred pathway in clonal evolution of uterine leiomyomas.

Present findings showed r(1) as the secondary change after translocation or insertion of chromosomes 12 and 14 in two tumors. Accompanied by this change in one myoma, in other cases ring chromosomes were detected in mosaic karyotype with a normal clone and/or with a clone of cells losing ring one.

4.2. Genome stability of UL

In order to investigate the role of HMGA proteins in genomic stability in a large series of UL, different groups of cells showing different levels of HMGA2 expression were considered and compared. Because of the low number of cases including 6p21 rearrangements and high expression of *HMGA1*, respectively, this analysis was performed just for the UL with 12q14~15 rearrangements (Fig. 4.1). Unstable condition for the cells was marked by metaphases including a structural single aberration or a chromosome/chromatid break.

Normal cells with an undetectable or detectable HMGA2 expression at very low level have a high genomic stability. As it is known malignant cells show various chromosomal aberrations with a reduction of genomic stability. If it would be accepted that HMGA2 impairs the integrity of genome and causes instability, therefore this group of cells with high genomic instability should have a very high expression of *HMGA2*, on the other hand, the stem cells with a high proliferation rate and extra expression of *HMGA2* will be expected to reveal relatively high instability in their genome. Furthermore, it is known for the benign cells including 12q14~15

rearrangements to have a high level of HMGA2 protein; therefore, this group of cells should also have a behavior like stem cells.

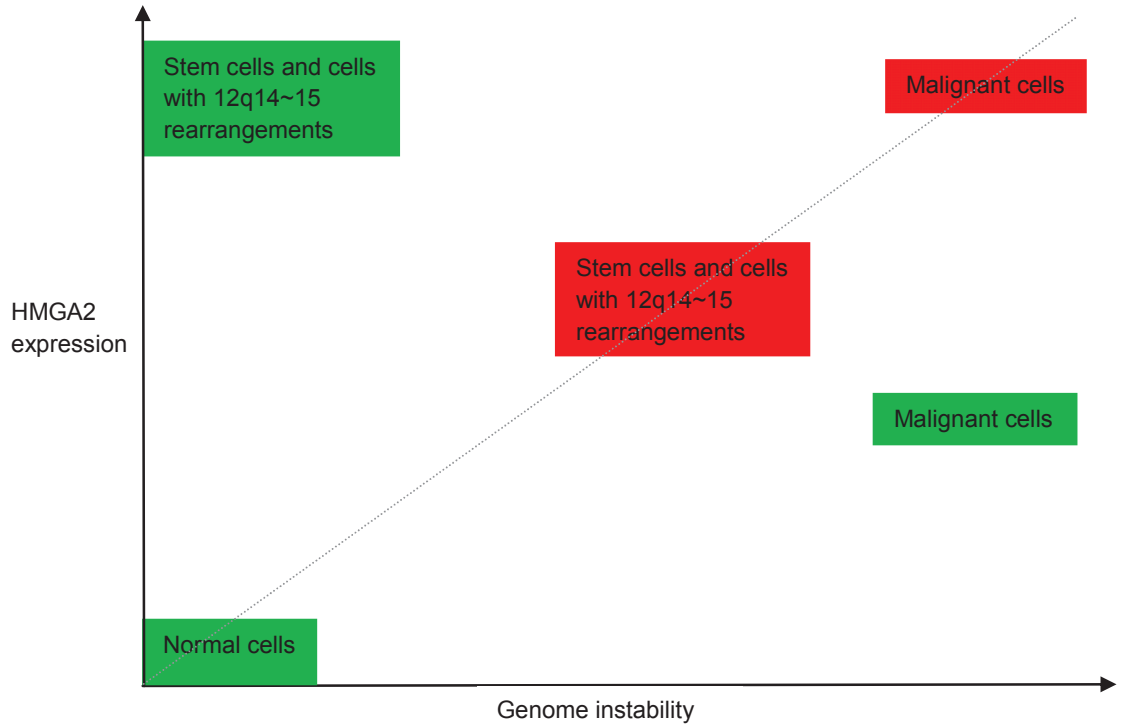


Fig. 4.1: Real and expected correlation of HMGA2 expression and genomic instability. Red boxes: expected place for each group; green boxes: real place for each group of cells.

To support the stated assumption some studies have linked HMGA proteins with an increased genomic instability due to a reduced capacity to repair DNA damage, for example inhibition of nucleotide excision repair by HMGA1 (Adair et al., 2005), repression of *ERCC1* gene by HMGA2 (Borrmann et al., 2003), and suppression of nonhomologous end joining (NHEJ) repair by overexpression of HMGA2 (Li et al., 2009). In a different cell type HMGA2 has been shown to bind directly to, and negatively regulate, the promoter of an important gene involved in the regulation of nucleotide excision repair (*ERCC1*) (Borrmann et al., 2003). Using the fibroblasts transfected by a construct encoding HMGA2 as a hallmark of deficient NHEJ, Li et al., (2009) argued that HMGA2 protein acts as an inhibitor of NHEJ. Through a reduction of DNA stability it facilitates the accumulation of chromosomal aberrations which is a central feature of tumorigenesis.

But the present results of the analysis of *HMGA2* expression using real time RT-PCR and cytogenetic investigation in these groups do not agree with this assumption. Investigations of three groups of myomas (UL showing rearrangements of 12q14~15 region without single aberration or chromosome/chromatid break (N=16), myomas with an apparently normal karyotype without single aberration or chromosome/chromatid break (N=151) , and all UL affected with single aberrations or chromosome/chromatid breaks (N=46)), revealed that there is no difference between the percentage of cells including a single structural aberration or chromosome/chromatid break between all UL with 12q14~15 rearrangements and all normal myomas. Considering the gene expression, there was no significant difference between affected group with a single structural aberration or chromosome/chromatid break and the UL without such changes, moreover there was no difference between the expression of *HMGA2* in affected UL with just one event and myomas including more than one chromosome/chromatid break or single aberration. In addition, it is proved the malignant cells with relatively higher levels of HMGA2 and known high genome instability; express *HMGA2* in a lower level than benign tumors including 12q14~15 rearrangements (Markowski et al., 2010b). It is true in stem cells also that this group of cells has a high expression of *HMGA2* despite their good genome stability.

In summary, stem cells and benign cells with involvement of 12q14~15, despite an overexpression of HMGA2, protect the stability of the genome well. In contrast, malignant cells with high genomic instability express HMGA2 in a lower level than benign and stem cells (Fig. 4.1). Bullerdiek and Rommel (2010) proposed that in this context a plausible assumption is that the cytogenetically unstable cells displaying sporadic translocations or dicentrics are those with strong overexpression of the recombinant HMGA2 in a range usually not found during embryonic development.

At the same time, it should not be forgotten that HMGA2 is a protein abundantly expressed during embryonic and fetal life (Rogalla et al., 1996; Li et al., 2007) and it is difficult to believe that this protein *per se* destabilizes the genome. On the other hand the benign behavior with rare malignant transformation in UL (Sandberg, 2005; Morton, 1998) and other benign tumors proves the high genomic stability in these cells.

For an explanation, it seems plausible to assume that the cells having 12q14~15 abnormalities protect the genome despite their high HMGA2. It might be the

existence of other factors accompanying high HMGA2 or its other role, which counterbalances its effect in the induction of genome instability.

Narita and colleagues introduced a novel role for HMGA proteins; these proteins also act in tumor suppressor networks by having a role in cellular senescence and heterochromatin formation (Narita et al., 2006). They showed that HMGA protein as a promoter of tumorigenesis joins $p16^{Ink4a}$ tumor suppressor in inducing proliferative arrest and senescence through their assistance in repressing proliferation-associated genes. Although in somatic stem cells, there is a link between HMGA2 and the *CDKN2A* locus (encoding *p16INK4a* and *p19Arf*) observed, the expression of this locus is related directly with cellular senescence control in most of the cell types and can be repressed by HMGA2 (Nishino et al., 2008). A recent investigation on the role of HMGA2 also supports the mentioned protection assumption, when another pathway correlated with HMGA2 in the growth and genome stability of UL was exhibited (Markowski et al., 2010a). This study revealed a high expression of senescence-associated $p19^{Arf}$ in the presence of overexpression of *HMGA2* in UL with 12q14~15 rearrangements. The results identify the $p19^{Arf}$-TP53-CDKN1A pathway as a balancer in the growth and genomic stability of UL in presence of a high level of HMGA2. The S phase of cell cycle is engaged in supporting the integrity of the genome and inhibiting genetic instability (Myung et al., 2001).

Therefore, the role of the known guardian of the genome, tumor suppressor protein p53 should be attended. p53, a transcription factor, encoded by the *TP53* gene, located on the short arm of chromosome 17 (17p13.1)(Matlashewski et al, 1984; McBride et al, 1986; Isobe et al., 1986; Kern et al., 1991), plays a role in apoptosis, senescence (an irreversible growth arrest), genetic stability, and inhibition of angiogenesis. The data by Izadpanah et al. (2008) presented that there is a coincidence between the arrest in the S phase of the cell cycle, detected in the long-term *in vitro* culture of human mesenchymal stem cells (MSCs) and the considerably suppressed expression of p53. Formerly, Bartek and Lukas (2001; 2003) admitted that S phase checkpoint mechanisms arrest the cell cycle in a p53-independent fashion.

Responding to DNA damage, p53 is able to inhibit the progression through the G1-S checkpoint in the cell cycle. In the case of prevalent DNA damage, it will activate the DNA repair proteins, and if the damage is not being repaired, p53 starts apoptosis. Therefore, the mutated cells are removed from the cell cycle by apoptosis, the

programmed cell death, and afterward, cell division will continue with health cells and the stability of genome will be protected well. Therefore, considering the stability of genome in the benign tumors with high expression of *HMGA2*, an intact p53 pathway is necessary.

4.3. Long-term expansion of myoma cells

For further investigations on myoma growth and HMGA proteins in the present study, a long-term *in vitro* expansion was performed on four UL with different HMGA levels: one with 12q14~15 rearrangement and very high level of HMGA2 (myoma 646), another one including 6p21 aberration and high HMGA1 expression (myoma 658.1), and in the same time for each case, one normal myoma as control (no. 641.2 and 659.2). All four cases, especially myoma 646 had in the beginning a fast proliferation pattern.

The cell cycle is a notably organized process which is result in for the loyal duplication and transmission of genetic information through the cell generations (Israels and Israels, 2001). *In vitro*, the primary mammalian cells are able to replicate to approximately 50 cumulative population doublings, after which the division of cells stops (Hayflick and Morrhead, 1961). This phenomenon is called Hayflick limit, and is widely distinguished as the replicative senescence. A basic step of quick proliferation can be seen in the proliferative lifespan of fibroblast cultures, which is succeeded by a phase of declining replicating frequency. The next stage is that of replicative senescence in which the cultures are not able to proliferate more (Vande Berg and Robson, 2003). The studies by Campisi et al. (1996); Faragher and Kipling (1998) came up with the assumption that the replicative senescence resulted from the limited lifespan of diploid cells is a key mechanism behind human aging *in vivo*. A proposed mechanism in regualting replicative senescence is the gradual erosion of chromosomal telomeres (telomere-dependent senescence) (Harley et al., 1990). The factor which can probably be of help in identifying the number of earlier cell divisions and thus, the replicative age of a considered cell, is the telomere erosion degree and the telomere length estimation (Harley et al., 1990); and additionally, as Baird et al., (2003b) confirms in his results, they can even help us find the potentiality of that cell to replicate further. Telomere shortening happens as the result of different processes, from which the end-replication case of DNA synthesis can be mentioned. This event can impress all cells lacking active telomerase (Enoch et al., 2009). Notable is to say

that; the somatic cells such as fibroblasts are not yet seen to express telomerase (Klapper et al., 2001), but tumor cells (Newbold, 2002) and stem cells (Wai, 2004) do, therefore it is probable that they do not encounter telomerase shortening which is by itself an introduction for indefinite replication.

A marked decrease in telomerase activity in progressively increasing passages of MSCs derived from the bone marrow and adipose tissue has previously been reported (Izadpanah et al., 2006). Loss of telomeres is discussed as one possible mechanism of chromosome instability (Blackburn, 2001).

It is mentioned that the cells of benign tumors undergo only a few divisions before senescence of the culture, but unbalanced chromosomal abnormalities cause an increased *in vitro* lifetime (Stern et al., 1990). The cells of UL show a limited and slow growth potential *in vitro* despite their high growth potential *in vivo* (Stern et al., 1991; Sandberg et al., 2005).

From considered samples, the normal UL grew until 14-15th P and then underwent replicative senescence. Both did not reveal any chromosomal aberration, just a few tetraploid metaphases (in a range of 2-4 metaphases in each objective slide) in the middle passages, but not in the first and the last of them in myoma 641.2. Interestingly, myoma 659.2, a normal case with different single cell aberration in the primary culture, did not show any aberration even tetraploidy either (Fig. 3.9).

Retaining the normal diploid karyotype in human mesenchymal stem cells in an extended culture was reported before (Izadpanah et al, 2008). According to their findings, a significant percentage of cycling human MSCs was arrested in S phase of the cell cycle at P20 and higher.

Tetraploidy, the result of abnormal mitotic division (Izadpanah et al., 2008), is not rare in fibroblast cultures (Bullerdiek und Rommel, 2010). It was also reported in the study by Izadpanah et al. (2008), in an expanded MSCs cultures. During further cell divisions, the cells lose their ability to maintain chromosome stability. Long-term cultures can lead to changes in cell cycle kinetics and produce a tetraploidy (Izadpanah et al., 2008). It has suggested that tetraploidy may result from endoreduplication, which has been associated with p53 inactivation (Vogel et al., 2004). This hypothesis was supported by introducing a p53-dependent pathway as an intrinsic capacity to eliminate tetraploid cells (Fujiwara et al., 2005; Shi and King, 2005).

Myoma 658.1 with karyotype formula: 46,XX,t(6;10)(p21;q22)[13]/46,XX,[8] and a very high expression of *HMGA1* grew up to P.25. This mosaic pattern was followed by the growth of only normal clone of cells after first passage. Any chromosomal alterations were observed in the long-term culture (Fig. 3.8) except teteraploid metaphases starting from P.5. The repression of aberrant cells by normal cells has been previously described (Stern et al., 1990).

Long-term culture of myoma 646 with 12q14~15 rearrangement and high *HMGA2* expression exhibited a near-tetraploid karyotype with 92 chromosomes in a few cells that started from P.11, changes of chromosome 9 started from P.12 by existence of der(9), monosomy of chromosome 6 in P.18 and later, and ring chromosome 9 in P.22 (Fig. 3.7). Derivative chromosome 9 and a monosomy of chromosome 6 in long-term culture of pleomorphic salivary gland adenomas were also observed by Stern et al. (1990). Ring chromosome 9 with appearance from P. 22 might be a transformation of the derivative chromosome 9 into a ring.

Presumably the new aberrations are caused by gradual adaptation of the cells to their culture conditions. Different factors were counted to contribute in these changes, such as: technique of chromosome preparation, incubation time of cells in cell culture until chromosome analysis, number and quality of the analysed metaphases (Stern, 1992), and culture medium, some extensive studies showed that the Chang-medium increases the chromosomal instability and various chromosome aberrations (Bui et al., 1984; Krawczun et al., 1989; Bartnitzke et al., 1992).

After a long-term *in vitro* culture, the spontaneous conversion of cells may affect the chromosomal stability of cells (Rubio et al., 2005; Miura et al., 2006). For the cells of myoma 646, it could be suggested that long life *in vitro* accompanied with other factors such as the existing 12q14~15 aberrations and high level of HMGA2 with an enormous mitotic division and telomere shortening has strongly promoted the chromosomal instability and further aberrations.

The behavior of the fibroids *in vitro* by a limited growth potential and lack of spontaneous immortalization was characterized (Stern et al., 1991; Carney et al., 2002). Further cultures of rapid proliferative myoma 646 proved this matter again. The cells underwent senescence in P. 26. Histopathology survey also demonstrated a typical leiomyoma pattern for this case. Another analysis on a series of UL including case no. 646, by Markowski et al. (2010b), explained the high proliferative rate of this sample despite its benign identity. This recent study has compared the

expression of *HMGA2* and senescence-associated p^{19Arf} in the tissue and matching cell culture cells of UL. Results revealed a marked decrease of the HMGA2 mRNA in culture in most cases with overexpression of HMGA2, and a clearly increased expression of p^{19Arf} associated with the decrease of HMGA2 in normal and 12q14~15 aberrant UL. This increased level of p^{19Arf} can be a good reason for fast senescence in both normal and aberrant myoma cells. In an exceptional condition, myoma 646 revealed a lower p^{19Arf} RQ value in culture rather than in tissue. This low level of senescence-associated protein can explain the rapid mitotic division in these cells.

Of note, recently recurrent mutations of MED12 have been identified in UL (Mäkinen et al., 2011) that seem to occur independent of 12q14~15 translocations (Markowski et al., 2012). Interestingly, these mutations have also been found in rare cases of leiomyosarcomas (Pérot et al., 2012; Markowski et al., 2013).

In conclusion, the acquired knowledge from this study could help to a better understanding of the biopathology, development and progression of fibroids. They are also a confirmation of the findings of the previous studies, while offering promoted knowledge concerning the pathogenesis of UL. Moreover, considering the widespread presence of HMGA proteins in various benign and malignant tumors, the present findings in addition to future investigations, can provide new approaches in the development of diagnosis and therapies of the tumors. The goal is to put fewer burden on patients and reduce the health system financial costs.

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May 2013, Bremen

7. Publications List

1. Markus Klemke, Anke Meyer, Maliheh Hashemi Nezhad, Sabine Bartnitzke, Norbert Drieschner, Christiane Frantzen, Ernst Heinrich Schmidt, Gazanfer Belge, Joern Bullerdiek. 2009. **Overexpression of *HMGA2* in uterine leiomyomas points to its general role for the pathogenesis of the disease**. GENES, CHROMOSOMES & CANCER 48:171–178.

2. Markus Klemke, Anke Meyer, Maliheh Hashemi Nezhad, Gazanfer Belge, Sabine Bartnitzke, Joern Bullerdiek. 2010. **Loss of let-7 binding sites resulting from truncations of the 3' untranslated region of HMGA2 mRNA in uterine leiomyomas**. Cancer Genetics and Cytogenetics 196: 119-123.

3. Dominique Nadine Markowski, Inga von Ahsen, Maliheh Hashemi Nezhad, Werner Wosniok, Burkhard Maria Helmke, Joern Bullerdiek. 2010. **HMGA2 and the $p19^{Arf}$-TP53-CDKN1A axis: A delicate balance in the growth of uterine leiomyomas**. GENES, CHROMOSOMES & CANCER 49: 661-668.

4. Maliheh Hashemi Nezhad, Norbert Drieschner, Sabrina Helms, Anke Meyer, Mahboobeh Tadayyon, Markus Klemke, Gazanfer Belge, Sabine Bartnitzke, Käte Burchardt, Christiane Frantzen, Ernst Heinrich Schmidt, Joern Bullerdiek. 2010. **6p21 rearrangements in uterine leiomyomas targeting *HMGA1***. Cancer Genetics and Cytogenetics 203: 247-252.

5. Maliheh Hashemi Nezhad, Sabine Bartnitzke, Joern Bullerdiek. 2011. **12q14~15 aberrations do not enhance the chromosomal instability in uterine fibroids.** Submitted.

I

Overexpression of *HMGA2* in uterine leiomyomas points to its general role for the pathogenesis of the disease

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Overexpression of *HMGA2* in Uterine Leiomyomas Points to its General Role for the Pathogenesis of the Disease

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An overexpression of *HMGA2* is supposed to be a key event in the genesis of leiomyoma with chromosomal rearrangements affecting the region 12q14-15 targeting the *HMGA2* gene, but gene expression data regarding differences between uterine leiomyomas with and those without 12q14-15 aberrations are insufficient. To address the question whether *HMGA2* is only upregulated in the 12q14-15 subgroup, the expression of *HMGA2* was analyzed in a comprehensive set of leiomyomas ($n = 180$) including tumors with 12q14-15 chromosomal aberrations ($n = 13$) and matching myometrial tissues ($n = 51$) by quantitative RT-PCR. The highest expression levels for *HMGA2* were observed in tumors with rearrangements affecting the region 12q14-15, but although *HMGA2* is expressed at lower levels in leiomyomas without such aberrations, the comparison between the expression in myomas and matching myometrial tissues indicates a general upregulation of *HMGA2* regardless of the presence or absence of such chromosomal abnormalities. The significant ($P < 0.05$) overexpression of *HMGA2* also in the group of fibroids without chromosomal aberrations of the 12q14-15 region suggests a general role of *HMGA2* in the development of the disease.

INTRODUCTION

Uterine leiomyomas (UL, fibroids) are the most frequent gynecological tumors and, despite being benign, constitute an enormous public health burden. Among the symptoms caused by uterine leiomyomas are menorrhagia, abdominal pain, and infertility (Stewart, 2001). Their actual prevalence is still a matter of debate and seems to vary among populations, but at least one third of women aged 30 years or older have one or more UL (Cramer and Patel, 1990; Baird et al., 2003; Heinemann et al., 2003). Their incidence seems to be higher in African American than in European American or European women (Marshall et al., 1998).

Currently, mutations of the two human genes encoding high mobility group proteins of the HMGA type, i.e., *HMGA1* and *HMGA2* have been assumed to be causally linked with the development of subsets of uterine leiomyomas. Both genes encode members of the so-called high mobility group proteins. HMGA proteins are capable of binding to the minor groove of AT-rich DNA with three DNA-binding domains (so-called AT-hooks), thus inducing conformational changes in chromatin structure and enabling the regulation of the expression of various target genes. In addition, they can interact with other proteins by means of their acidic domain (Fusco and Fedele, 2007). *HMGA1* and *HMGA2* map to chromosomal bands that are targeted by nonrandom structural chromosomal abnormalities found in uterine leiomyomas, i.e., 6p21 for *HMGA1* (Kazmierczak et al., 1996) and 12q14-15 for *HMGA2* (Ashar et al., 1995; Schoenmakers et al., 1995). Usually, these regions are affected by chromosomal translocations but inversions can occur as well with structural chromosomal aberrations affecting 12q14-15 being much more frequent than those affecting 6p21 (Nilbert and Heim, 1990). The molecular alterations resulting from the cytogenetic deviations generally seem to

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include an upregulation of the genes (Tallini et al., 2000; Gross et al., 2003). With regard to *HMGA2*, a considerable fraction of the chromosomal breakpoints has been assigned to regions outside the open reading frame of the gene, thus primarily affecting its expression rather than its protein sequence (Quade et al., 2003). Thus, overexpression of *HMGA2* seems to be sufficient to trigger tumorigenesis. It is obvious that an enhanced level of *HMGA2* is pathogenetically relevant in a subset of some 10–20% of uterine leiomyomas (Hennig et al., 1999), but it remains an open question whether or not an increased level of *HMGA2*, compared to normal myometrium, may characterize also leiomyomas that do not have *HMGA2* rearrangements. A recent study by Peng et al., (2008) suggests that upregulation of *HMGA2* may be a more general phenomenon in UL but the analyzed tumors were not genetically classified and no matched samples were analyzed individually by quantitative RT-PCR. Herein, we have quantitated the level of *HMGA2* mRNA in a large series of uterine leiomyomas.

MATERIALS AND METHODS

Tissue Samples

Samples of uterine leiomyomas and myometrium were snap frozen in liquid nitrogen immediately after surgery and stored at −80°C. In case of UL another part of the tumor was used for cell culturing and karyotyping. Informed consent was obtained from all patients.

For fluorescence in situ hybridization (FISH) analyses HOPE (HEPES-glutamic acid buffer mediated organic solvent protection effect)-fixed, paraffin-embedded tissue sections were used. The tissues were cut into 5 μm sections which were subsequently used for FISH analyses.

RNA Isolation, Reverse Transcription, and Quantitative RT-PCR

RNA was isolated from fresh-frozen tissue samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany) including DNase treatment according to the manufacturer's instructions and quantitated by spectrophotometry. After reverse transcription of 250 ng of total RNA using M-MLV RT (Invitrogen, Karlsruhe, Germany) and random hexamers, the *HMGA2* mRNA levels were determined by relative quantification referring to the expression of 18S rRNA. Real-time PCR was performed on a 7300 Real-Time PCR System with Assay No. Hs00171569_m1 (Applied Biosystems, Darmstadt, Germany) for the detection of *HMGA2* and primers and probe for 18S rRNA as described previously (Belge et al., 2008).

Analysis of Gene Expression

The relative expression was calculated by the ΔC_t method, using 18S rRNA as endogenous control and by choosing the *HMGA2* expression of a myometrial sample as calibrator. The significance of differential *HMGA2* expression between the different groups (myometrium, myoma with and without 12q14-15 aberrations) was determined by Student's *t*-test.

Cell Culture

After surgery, samples of primary tumors were stored in Hank's solution with antibiotics (200 IU/ml penicillin, 200 μg/ml streptomycin). For cell culture the tumor samples were minced and treated with 0.26% (200U/ml) collagenase (Serva, Heidelberg, Germany) for 5–8 hr. After centrifugation, the pellet was resuspended in culture medium (TC 199 with Earle's salts supplemented with 20% fetal bovine serum, 200 IU/ml penicillin, 200 μg/ml streptomycin) and incubated at 37°C and 5% CO_2.

Chromosome Analyses

For chromosome analyses exponentially growing cultures of leiomyoma cells were used. Metaphase chromosome spreads were prepared by using colcemid (0.06 μg/ml for 1 hr) to arrest cultured cells during mitosis. A hypotonic solution (culture medium and aqua bidest in a 1:6 ratio) and the fixative (methanol and acetic acid in a 3:1 ratio) were then applied sequentially. Finally, the chromosome suspension was dropped onto glass slides. The chromosomes were GTG-banded according to routine techniques. Karyotype description followed ISCN (2005).

HMGA2-Specific Break-Apart Probes and FISH

For FISH three BAC clones were used as break-apart probes. RP11-745O10 (AC078927) and RP11-293H23 (AC012264) are located distal (3′) to *HMGA2*. RP11-269K4 (AQ478964 and AZ516203) is located proximal (5′) to *HMGA2*. Labeling was performed by nick translation (Roche Diagnostics, Mannheim, Germany) either with digoxigenin (RP-269K4) or biotin (RP11-

745O10 and RP11-293H23). For each FISH experiment 2 ng/μl of the distally located probes (RP11-745O10 and RP11-293H23) and 3 ng/μl of RP11-296K4 were used in 15 μl hybridization solution containing 50% formamide, 2×SSC, 10% dextrane sulfate and 105 ng/μl COT human DNA.

FISH analysis on metaphase preparations was performed after GTG banding of the metaphase spreads. Treatment of metaphases and subsequent FISH experiments were performed as described previously (Kievits et al., 1990) with a few modifications. For one slide 25 μl of hybridization mixture were used. Codenaturation was performed on a Mastercycler gradient (Eppendorf, Hamburg, Germany) for 3 min at 80°C followed by O/N hybridization in a humidified chamber at 37°C. Posthybridization was performed at 61°C for 5 min in 0.1×SSC. Subsequent treatment of slides was performed as described previously (Kievits et al., 1990). For detection of the hybridized probes antidigoxigenin fluorescein fab fragments (Roche Diagnostics) and Cy3-conjugated streptavidin (Dianova, Hamburg, Germany) were used. Slides were counterstained with DAPI (0.75 μg/ml) (Vector Laboratories, Burlingame, CA).

For FISH, formalin-fixed, paraffin-embedded (FFPE) tissue sections were deparaffinized with diethylether. Protease digestion was done with a pepsin ready-to-use solution (DCS, Hamburg, Germany) for 12–17 min. After dehydration in a 70%, 80%, and 95% ethanol series the sections were postfixed with 1% formaldehyde in 1×PBS for 15 min. Prior to codenaturation the sections were dehydrated again. Codenaturation was performed on a Mastercycler gradient (Eppendorf) for 5 min at 85°C followed by O/N hybridization in a humidified chamber at 37°C. Posthybridization was performed at 42°C or 61°C for 2 min in 0.4×SSC/0.3%NP-40. Subsequent treatment of slides and detection of hybridized probes were performed as described for FISH on metaphase preparations.

Slides were examined in an Axioskop 2 plus fluorescence microscope (Zeiss, Göttingen, Germany). Images were captured with an AxioCam MRm digital camera and were edited with AxioVision (Zeiss). For metaphase preparations, 10 metaphases were examined. For analysis of FFPE tissue sections at least 100 nonoverlapping nuclei from different (at least three) areas of the tumors were scored. Nuclei with two colocalized red/green signals (RG) were scored as normal. Nuclei with one colocalized red/green signal, one single red, and one single green signal (1RG1R1G) were scored as positive for *HMGA2* rearrangement.

RESULTS

For this study 180 uterine leiomyomas from 100 patients have been investigated by qRT-PCR for the expression level of *HMGA2*. A total of 57 myometrium samples from uteri removed because of the occurrence of UL were investigated as well. For 51 of these samples, matching tissue from one or more leiomyomas was available. All UL have been karyotyped successfully based on at least 10 G-banded metaphases showing a resolution of 400 bands per haploid set or higher.

Based on cytogenetics the group of UL was further subdivided into those showing aberrations of chromosomal region 12q14-15 ($n = 13$; Table 1) and those with an apparently normal karyotype or other clonal aberrations ($n = 167$), respectively.

As to these three groups, i.e., myometrium, UL with 12q14-15 changes, and other UL *HMGA2* expression was determined by qRT-PCR using fresh-frozen samples. The average relative *HMGA2* mRNA expression was 1.99 for the myometria and 261.41 for all UL. When distinguishing between both subgroups of UL outlined above average expression levels were 3213.78 for UL with aberrations in the chromosomal region 12q14-15 and 31.59 for those without changes in this region, respectively.

Thus, even within the group of UL without cytogenetically detectable rearrangements of the *HMGA2* locus at 12q14-15 *HMGA2* mRNA was expressed at a higher level than in myometrium (Fig. 1). Differences between all leiomyomas and myometrium as well as between leiomyomas without 12q14-15 aberrations and myometrium were statistically significant ($P < 0.005$ and $P < 0.05$, respectively). Furthermore, an individual analysis of the matched samples (51 myometrial tissues and 107 corresponding UL) was performed. The mean *HMGA2* expression was 11.37 in karyotypically normal UL ($n = 101$) and 1.77 in the corresponding myometrial tissues ($n = 51$).

The results clearly show that in nearly all cases within each of the paired samples the leiomyomas showed higher *HMGA2* expression than the corresponding myometrium (Fig. 2).

One case with a normal karyotype and an unexpectedly high *HMGA2* expression as well as

TABLE 1. Karyotypes of the 13 Leiomyomas with Chromosome 12 Aberrations and Results of Interphase FISH with *HMGA2* Specific Break-Apart Probes

Karyotype	Relative *HMGA2* expression	FISH results (2RG/1RG1R1G)[a]
46,XX,inv(5)(q15q31∼ 33),t(12;14)(q15;q24)[13]	8.6	98/1
46,XX,t(12;15;14)(q15;q26;q24)[20]/46,XX[1]	302.3	–
46,XX[36]	894.5	72/23
–	993.3	41/51
46,XX,der(1)r(1;2),t(12;14)(q15;q24)[4]/46,XX,t(12;14)(q15;q24)[13]	1047.8	m
46,XX,t(12;14)(q15;q24)[9]/46,XX[3]	1327.3	–
46,XX,r(1),t(1;12;14)(p36.3;q14;q24)[19]	1722.5	–
46,XX,t(2;12)(q33;q13)[17]	2381.6	–
46,XX,der(12),der(14)?ins(14;12)[8]/46,idem,r(1)[4]	3444.3	–
46,XX,t(3;5;12)(q23∼ 25;p13∼ 15;q13∼ 15)[11]/45,XX,idem,-22 [10]	4450.7	–
46,XX,t(12;14)(q15;q24)[5]/46,XX[9]	5906.1	–
45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),-22[15]	7760.3	–
45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),-22[15]	11539.7	–

[a]Percentage of nuclei either with two colocalized signals (2RG) or with one colocalized, one single red and one single green signal (1RG1R1G) indicating a *HMGA2* rearrangement.
m, FISH was performed on metaphase preparations (Fig. 4). All 10 metaphases showed a breakpoint upstream of *HMGA2*.

a second myoma with a cytogenetically visible t(12;14) and a rather low *HMGA2* expression were checked by interphase FISH. In the cytogenetically normal myoma, FISH showed *HMGA2* disruption in 23% of the cells (Fig. 3, Table 1). The tumor with visible t(12;14) but low expression of *HMGA2* showed two colocalized signals in 98% of the nuclei, indicating an intact *HMGA2* locus.

Metaphase FISH was also done on one case with a t(12;14)(q15;q24) (Fig. 4). Interestingly, the probe located proximal to *HMGA2* (RP11-269K4) showed three signals: on the normal chromosome 12, the derivative chromosome 12, and the derivative chromosome 14 (Fig. 4C), indicating a breakpoint located 5′ of *HMGA2*. The approximately 16kb distance between the probe RP11-269K4 and the 5′ end of *HMGA2* suggests that the breakpoint was located approximately 20kb upstream of *HMGA2*.

DISCUSSION

Despite their high prevalence the etiology and pathogenesis of UL remain poorly understood. Mutations of the gene encoding the high mobility group protein HMGA2 have been suggested to cause a subset of uterine leiomyomas (Schoenmakers et al., 1995; Hennig et al., 1999). As a

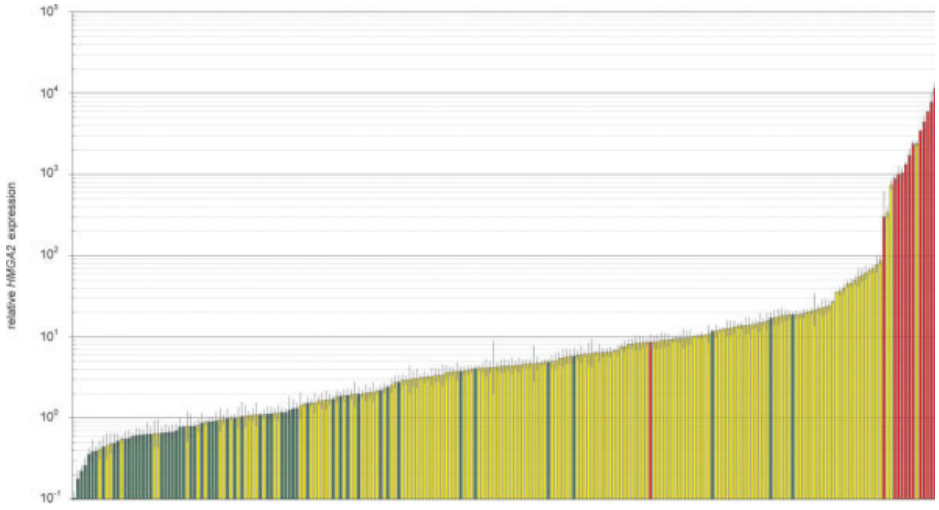

Figure 1. Relative quantification of the *HMGA2* expression in uterine leiomyomas and myometrial tissues. Green bars: Myometrium; yellow bars: UL without cytogenetically detectable aberrations of chromosomal region 12q14-15; red bars: UL with 12q14-15 aberrations.

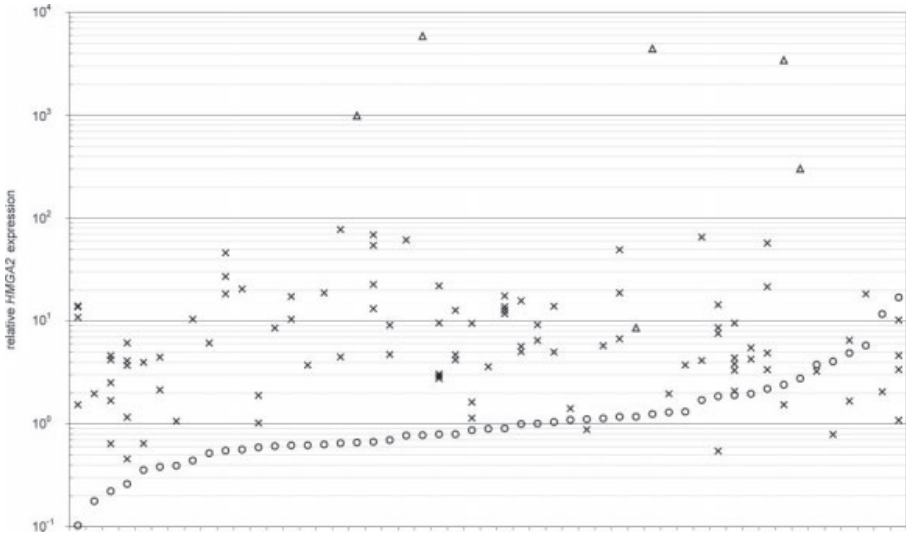

Figure 2. *HMGA2* expression in UL (crosses) and matching myometrial tissues (open circles) in increasing order of expression in myometrial tissues. Triangles indicate UL with 12q14-15 aberrations.

rule, this subset is characterized by cytogenetically visible structural chromosome alterations affecting chromosomal region 12q14-15. *HMGA2* has been identified as the target of these alterations (Schoenmakers et al., 1995) and despite a wide distribution of breakpoints, intragenic as well as extragenic (Kazmierczak et al., 1995; Hennig et al., 1996; Schoenmakers et al., 1999; Kurose et al., 2000; Mine et al., 2001; Takahashi et al., 2001; Quade et al., 2003), the key mechanism by which the chromosomal alterations contribute to tumorigenesis seems to be an upregulation of the *HMGA2* gene leading to overexpression of the full-length transcript or a truncated or chimeric protein. Nevertheless, the majority of UL lack cytogenetically visible chromosome alterations and also by molecular-cytogenetic methods there is no evidence that submicroscopic alterations of the *HMGA2* locus occur in a considerable number of these cases (Weremowicz and Morton, 1999). On the other hand, *HMGA2* overexpression could play a more general role in the development of UL, and not only in the subgroup characterized by 12q14-15 alterations. Roughly 10 years ago the hypothesis was advanced that *HMGA2* overexpression induces an embryonic chromatin configuration in cells, thus re-endowing them with a stem-cell like behavior (Bullerdiek, 1997). This assumption was further supported by recent studies on the *HMGA2* expression in embryonic stem cells (Li et al., 2006, 2007).

Here, we have shown that also UL without cytogenetically detectable 12q14-15 rearrangements overexpress *HMGA2*. This supports the assumption that an elevated level of a stem-cell chromatin associated protein is one of the key events in the genesis of UL. Of particular note, the expression of *HMGA2* in myomas almost always exceeded that of the corresponding myometrium (Fig. 2). Apparently, the basic level of *HMGA2* varies among the samples. Possibly, this could reflect changes throughout the menstrual

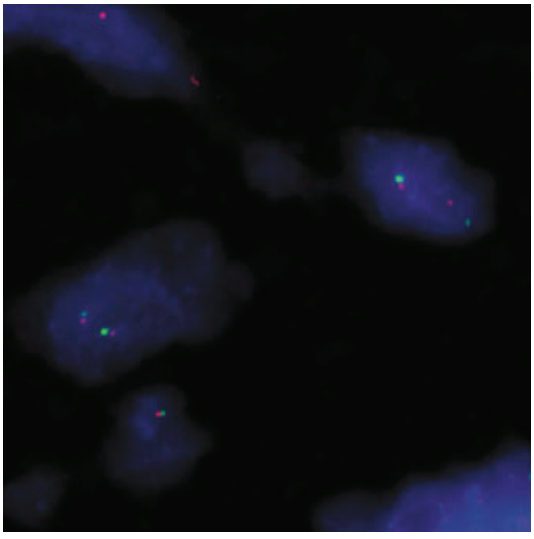

Figure 3. Dual-color FISH performed on interphase nuclei of a leiomyoma with cytogenetically normal karyotype. One nucleus showing two colocalized red/green signals (2RG, left) and a second nucleus with one colocalized red/green and one single red and green signal (1RG1R1G, right), respectively, indicating a rearrangement of *HMGA2*.

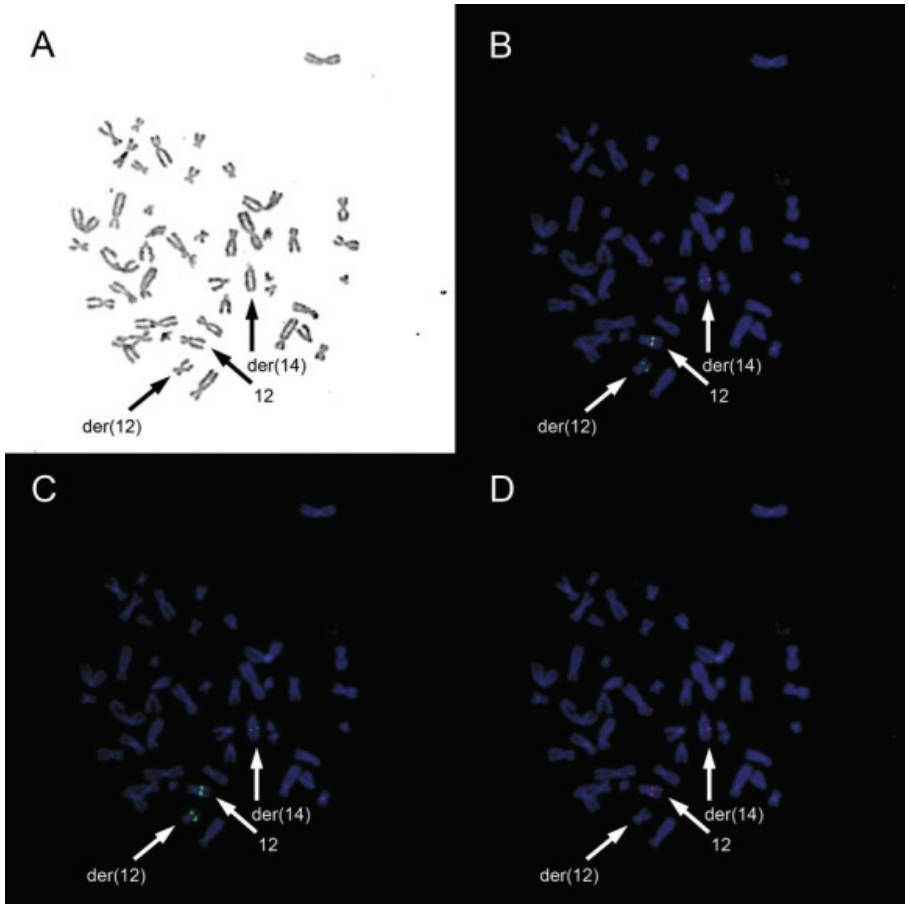

Figure 4. FISH with *HMGA2* break-apart probes in a UL with t(12;14)(q15;q24). A: G-banded metaphase prior to FISH. B: The same metaphase after dual-color FISH indicating a rearrangement of the *HMGA2* locus. C: Corresponding figure only showing the green fluorescent probe (RP11-269K4) located proximal (5′) to *HMGA2*. D: Corresponding figure only showing the red fluorescent probes (RP11-745O10 and RP11-293H23) located distal (3′) to *HMGA2*.

cycle. Such alterations in gene expression patterns have been described, e.g., by Kayisli et al. (2007). Herein, the term overexpression refers to an expression exceeding that of the matching myometrium. Despite the monoclonal origin of UL (Townsend et al., 1970; Mashal et al., 1994; Hashimoto et al., 1995; Zhang et al., 2006) overexpression of *HMGA2* must not necessarily be due to mutations affecting the gene itself. It has recently been described that *HMGA2* is regulated by microRNAs of the let-7 family (Lee and Dutta, 2007; Mayr et al., 2007; Park et al., 2007; Shell et al., 2007; Kumar et al., 2008; Motoyama et al., 2008; Peng et al., 2008). Thus, having now identified the overexpression of *HMGA2* also in UL without 12q14-15 alterations being visible at the microscopic level, future studies should also address mutations of let-7 genes and their binding sites within the 3′ UTR of *HMGA2*.

To further validate the cytogenetic results in cases with high *HMGA2* expression and no visible translocation or vice versa, FISH was performed with *HMGA2* break-apart probes. In the first case, separate signals from BAC clones located 5′ and 3′ of *HMGA2* occurred in 23% of the cells, indicating a translocation with a breakpoint within or in close proximity of *HMGA2*. Besides a cryptic *HMGA2* rearrangement undetectable by classical cytogenetics, a selection of cells without translocation during cell culture may explain the observation that the karyotype was apparently normal. However, the fact that chromosome 12 was found to be aberrant in almost one fourth of the cells by FISH is concordant with the high *HMGA2* expression despite an apparently normal karyotype.

In the second case showing a low *HMGA2* expression despite a visible t(12;14) the signals

were colocalized in 98% of the cells suggesting that the breakpoint was localized outside the region covered by the FISH probes. This unusually large distance between the breakpoint and *HMGA2* may explain why the observed *HMGA2* expression was unexpectedly low in one UL with a t(12;14). On the other hand, as indicated by FISH on metaphases of one UL with t(12;14)(q15;q24), an extragenic breakpoint upstream but in closer proximity of *HMGA2* can be sufficient to trigger the observed overexpression. Breakpoints located 5′ of *HMGA2* in UL with t(12;14) have also been reported by Quade et al. (2003).

The *HMGA2* expression in uterine leiomyomas has also been quantified in a study by Gross et al. (2003). Eleven karyotypically normal UL plus four matching myometrial samples as well as 10 UL with 12q14-15 rearrangements plus three myometrial tissues were analyzed by qRT-PCR, and a significantly higher *HMGA2* expression in UL with 12q14-15 rearrangements was noted. However, in contrast to the present study, no significant differences between the expression levels in karyotypically normal UL and matching myometrial samples were observed.

In summary, our results confirm the strongly increased *HMGA2* expression in UL with 12q14-15 rearrangements. Moreover, the expression levels detected in 101 UL from 51 patients and 51 matching myometrial samples indicate a general increase of *HMGA2* mRNA also in karyotypically normal tumors.

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II

Loss of let-7 binding sites resulting from truncations of the 3' untranslated region of HMGA2 mRNA in uterine leiomyomas

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Loss of *let-7* binding sites resulting from truncations of the 3' untranslated region of *HMGA2* mRNA in uterine leiomyomas

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Abstract A subset of uterine leiomyomas (UL) shows chromosomal rearrangements of the region 12q14~q15, leading to an overexpression of the high-mobility group protein A2 gene (*HMGA2*). Recent studies identified microRNAs of the *let-7* family as post-transcriptional regulators of *HMGA2*. Intragenic chromosomal breakpoints might cause truncated *HMGA2* transcripts lacking part of the 3' UTR. The corresponding loss of *let-7* complementary sites (LCS) located in the 3' UTR would therefore stabilize *HMGA2* mRNA. The aim of this study was to check UL with rearrangements of the chromosomal region 12q14~15 for truncated *HMGA2* transcripts by real-time reverse-transcription polymerase chain reaction. In 8/13 leiomyomas with aberrations of chromosomal region 12q15, the results showed the presence of the complete 3' UTR with all LCS. A differential expression with highly reduced 3' untranslated region levels was found in 5/13 myomas. In two of these, full-length transcripts were almost undetectable. Truncated transcripts were apparently predominant in roughly one-third of UL with chromosomal rearrangements affecting the *HMGA2* locus, where they lead to a higher stability of its transcripts and subsequently contribute to the overexpression of the protein. The assay used is also generally suited to detect submicroscopic alterations leading to truncated transcripts of *HMGA2*.

1. Introduction

A subgroup of uterine leiomyomas (UL) is characterized by rearrangements of chromosomal segment 12q14~q15. Roughly 15 years ago, a causal link among these aberrations, the deregulation of the gene encoding the high-mobility group AT-hook 2 (*HMGA2*), and the pathogenesis of UL has been shown [1–3]. *HMGA2* was found to be targeted by breakpoints that are located either intragenically or extragenically 3' or 5' of the gene. This type of abnormality is shared by a variety of other mainly benign tumors of mesenchymal origin (e.g., lipomas or pulmonary chondroid hamartomas) [1,2,4,5]. Initially, the transcriptional deregulation of *HMGA2* by the rearrangements of controlling elements and/or fusion genes was thought to be the relevant molecular alteration resulting from the chromosomal rearrangements. Because *HMGA2* is abundantly expressed during prenatal development and because its rearrangements often leave the open reading frame (ORF) intact, it is tempting to assume that the increased protein level alone is sufficient to cause or contribute to UL development [6]. Accordingly, the 12q14~q15 rearrangements are always associated with a drastically increased level of *HMGA2* mRNA [7–9]. However, there have been recent descriptions of another mechanism by which the chromosomal deviations can lead to a higher stability of the *HMGA2* transcript and, subsequently, a higher protein level as well. The 3' untranslated region (UTR) of *HMGA2* was shown to harbor multiple binding sites for microRNAs of the *let-7* family [10–14]. Truncations of the *HMGA2* transcript resulting from intragenic breakpoints can thus reduce the sensitivity of the transcript against microRNAs of the *let-7* family, finally leading to a higher protein level in the corresponding cells. This also explains an earlier observation that constructs containing a truncated *HMGA2* 3' UTR are more stable than those with a wild-type UTR [15]. While this mechanism is experimentally well documented, it is unclear to which extent truncation of *HMGA2*

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mRNA coincides with the chromosomal rearrangements of the gene locus and could amplify the effect of simple transcriptional up-regulation. To address this question, we have quantified and compared the level of wild-type and truncated *HMGA2* mRNA from 13 primary UL and 2 cell lines derived from UL with 12q14~q15 rearrangements by real-time reverse-transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Tumor samples and cell culture

Tissue samples of uterine leiomyomas were collected immediately after surgery, snap-frozen in liquid nitrogen, and stored at −80 °C for RNA isolation. Samples used for cell culturing were treated as described previously [9].

Immortalized cells from a lipoma with a t(3;12)(q27~q28;q14~q15) accompanied by a partial genomic deletion of the *HMGA2* locus were used as a control because the presence of fusion transcripts consisting of exons 1–3 of *HMGA2* and exons 9–11 of *LPP* was shown for this case [16]. Cells were cultured in medium 199 with Earle's salts containing 1% fetal calf serum (FCS) for 24 hours and in FCS-free medium for an additional 24 hours to avoid the stimulating effects of FCS on the expression of *HMGA2* from the nonrearranged allele. Before the isolation of total RNA, cells were lysed directly in the cell culture flask.

2.2. Methods

Total RNA was isolated from tissue samples and cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany). In addition to the on-column DNase I digestion, a second digestion was performed in solution before reverse transcription, since the PCR reaction with the primer set binding in the 3' UTR of *HMGA2* is highly sensitive to contaminations with genomic DNA.

Reverse transcription of 250 ng RNA was carried out with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Controls without enzyme (NoRT) were included for each sample to ensure the absence of DNA contaminations, which would introduce a bias to the results of the 3' UTR–specific primer set.

Quantitative real-time RT-PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with TaqMan Universal PCR Mastermix. Of each cDNA, 2 μL served as template in a final reaction volume of 20 μL. Reaction conditions were as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, and 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. A commercial *HMGA2*-specific assay (assay ID Hs00171569_m1; Applied Biosystems) with primers binding in exons 1 and 2 was used to detect transcripts irrespectively of a possible downstream truncation. In addition, for full-length transcripts with an intact 3' UTR, a set of primers and probe complementary to the distal 3' end of the mRNA downstream of all *let-7* complementary sites (Fig. 1) was designed (forward primer: 5'-TGTATTAT CACTGTCTGTTCTGCACAA-3', reverse primer: 5'-TGG AACTGTAACAAAGAGCAGGAA-3', probe: 6FAM-CAG CCTCTGTGATCCCCATGTGTTTTG-TAMRA). A differential expression of full-length and truncated *HMGA2* transcripts has been reported recently for lipomas with t(3;12) [17]. Herein, for evaluating the 3' UTR–specific primer/probe set and graphic display of the results, the same method with slight modifications has been used. To evaluate the primer and probe set designed for the distal 3' UTR of *HMGA2*, a cell line of a lipoma with t(3;12) was tested for differential expression. In a previous study [16], we had reported the presence of *HMGA2-LPP* fusion transcripts consisting of exons 1–3 of *HMGA2* and exons 9–11 of *LPP* in this cell line.

HPRT1 was chosen as an endogenous control and was detected with the following primer/probe set: forward primer: 5'-GGCAGTATAATCCAAAGATGGTCAA-3', reverse primer: 5'-GTCTGGCTTATATCCAACACTTCGT-3', probe: 6FAM-CAAGCTTGCTGGTGAAAAGGACCCC-TAMRA. All reactions were run in triplicate. Due to the low *HMGA2* expression in normal tissues, the Ct values of the 3' UTR–specific PCR were much higher in myometrial tissues than in myomas with aberrations affecting the chromosomal region 12q15, making the use of normal tissue as a calibrator unsuitable. Therefore, the myoma with the smallest difference in dCt values between both PCRs (case 4) was chosen as a calibrator. Although the chromosomal region 12q13~q15 is rearranged in this case, the closely related dCt values indicate the predominance of full-length transcripts. Thus, an overestimation of the relative 3' UTR expression resulting from the alternative use of myometrial tissues was avoided. The $\log_{10}$ of the relative expression was used for graphic display (Fig. 2).

3. Results

In a total of 234 uterine leiomyomas from 124 patients, chromosome analysis of GTG-banded metaphases revealed rearrangements of the chromosomal region 12q14~15 in 12 cases (Table 1), which were subjected to real-time RT-PCR analysis. One additional myoma (case 13) had an apparently normal karyotype, but fluorescence *in situ*

Fig. 1. Schematic representation of the full-length *HMGA2* transcript with the ORF consisting of five exons (gray boxes) and *let-7* complementary sites (LCS) in the 3' UTR (black lines) according to TargetScan [20], PicTar [21], and miRanda [22,23]. For a detailed list of the LCS, see ref. 11, Table S2. The black bars below the transcript indicate the positions of the regions amplified with two different primer sets.

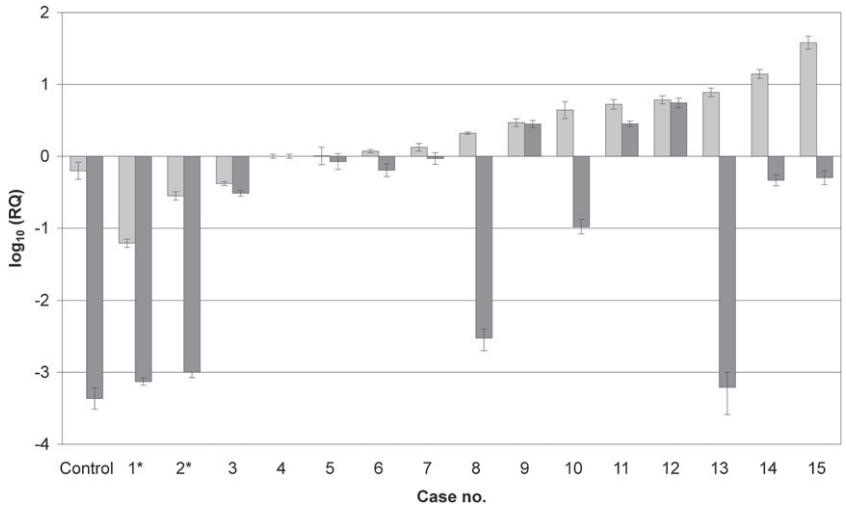

Fig. 2. Relative expression of *HMGA2* in uterine leiomyomas with chromosomal aberrations affecting region 12q14~15 and a lipoma cell line with known absence of full-length transcripts as a control. The $\log_{10}$ of the relative expression levels is shown (RQ: relative quantification). qRT-PCR was performed with primers located in exons 1 and 2 of *HMGA2* (light gray bars) and an additional set of primers located in the distal 3' UTR of the gene (dark gray bars). Case 4 was used as calibrator. Asterisks indicate cell lines of myomas with t(12;14).

hybridization (FISH) revealed split signals for *HMGA2*, indicating a hidden rearrangement of the gene locus.

With the criteria defined by Bartuma et al. [17] (i.e., an expression level that is 10 times higher for exons 1 and 2 than for the distal 3' UTR), a differential expression was observed in two myoma cell lines (cases 1 and 2), as well as in 5/13 myomas (cases 8, 10, 13, 14, and 15; Fig. 2). In contrast, no differential expression was detected in the remaining eight myomas.

In the lipoma cell line, which was used as control, the relative expression of *HMGA2* exons 1–2 was only slightly lower than in the myoma used as the calibrator, but the expression of the distal 3' UTR is clearly reduced ($\log_{10} = -3{,}36$), thus indicating the usefulness of the test procedure used.

4. Discussion

We recently reported an overexpression of *HMGA2* in uterine leiomyomas with a normal 46,XX karyotype in comparison to matching myometrial tissues [9]. Moreover, our results confirmed the previous finding, that *HMGA2* is strongly overexpressed in leiomyomas with chromosomal aberrations, affecting the locus of the gene [7–9]. However, the molecular mechanisms causing an overexpression, especially in those cases with cytogenetically detectable translocations with breakpoints in or close to chromosomal region 12q14~15, remain to be identified. Several studies [10–13] have reported the post-transcriptional regulation of *HMGA2* expression by miRNAs of the *let-7* family. Reduced expression of *let-7* family members in uterine

Table 1
Karyotypes of 13 leiomyomas and two cell lines (nos. 1 and 2) originating from myomas with chromosomal translocations affecting region 12q13~q15

Case no.	Karyotype	Age (yr)	Tumor diameter (cm)
1	46,XX,del(7)(q22q32),t(12;14)(q15;q24)[29]	–	–
2	46,X,t(X;12)(q22;q15)[8]	–	–
3	46,XX,t(12;15;14)(q15;q26;q24)[20]/46,XX[1]	49	1.5
4	46,XX,t(3;5;12)(q23~25;p13~15;q13~15)[11]/45,XX,idem,–22[10]	42	5.0
5	46,XX,der(7)del(7)(p)del(7)(q),add(8)(q2?),add(10)(q2?),t(12;14)(q15;q24)[15]	40	8.0
6	46,XX,t(12;14)(q15;q24)[9]/46,XX[3]	47	5.0
7	46,XX,r(1),t(1;12;14)(p36.3;q14;q24)[7]	40	6.0
8	46,XX,t(2;12)(q33;q13)[17]	49	10.0
9[a]	n. a.	46	3.0
10	46,XX,t(12;14)(q15;q24)[12]/ 45,XX,der(1),?t(1;2),–2,add(7)(?q36),t(12;14)(q15;q24)[2]/ 46,XX,del(4)(q31~q32),der(10),?t(10,14)(q24;q32),t(12;14)(q15;q24)[9]	47	4.0
11	46,XX,t(12;14)(q15;q24)[5]/46,XX[9]	37	7.0
12	45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),–22[8]	32	10.0
13[a]	46,XX	48	1.5
14	46,XX,t(12;14)(q15;q24)[13]/46,XX,der(1)r(1;2),t(12;14)(q15;q24)[4]	44	6.0
15	46,XX,ins(14;12)[8]/46,idem,r(1)[4]	73	2.5

Abbreviation: n.a., not available

[a] Case in which FISH revealed split signals for the *HMGA2* locus, indicating a rearrangement of the gene.

leiomyomas has been reported as well [14]. In addition to a downregulation of miRNAs responsible for the repression of *HMGA2* expression, losses of the *let-7* complementary sites (LCS) within the 3' UTR of *HMGA2* may also lead to a deficiency in *let-7*–mediated regulation [10]. Herein, we have investigated 13 leiomyomas as well as two cell lines of myomas with chromosomal aberrations affecting the *HMGA2* locus using a real-time RT-PCR approach to detect the truncation of *HMGA2* transcripts.

In 8/13 myomas, no differential expression between *HMGA2* exons 1 and 2 and the 3' UTR was observed (Fig. 2). This is in good agreement with previous studies reporting breakpoints upstream of the *HMGA2* gene in leiomyomas with rearrangements of chromosomal region 12q14~15 [18].

A lipoma with a t(3;12)(q27~q28;q14~q15) and a known disruption of the *HMGA2* gene was used as a control. The observed expression of the 3' UTR is extremely low in this sample, indicating the near absence of full-length transcripts. A complete absence should lead to negative PCR results, however, the negligible expression level is likely to be caused by a minor transcription of the unaltered allele.

Both cell lines as well as 5/13 myomas revealed a differential expression of exons 1–2 and the 3' UTR. In three of these tumors (cases 10, 14, and 15), however, a noteworthy amount of transcripts is truncated, but since full-length transcripts do not seem to be absent, the chromosomal breakpoint is unlikely to be located within the gene.

Two myomas (nos. 8 and 13) revealed expression levels of the 3' UTR, which are comparably low as the 3' UTR expression in the control, indicating the almost complete absence of full-length transcripts. Of note, the karyotype of one of these cases (no. 13) is apparently normal, but FISH performed on interphase nuclei indicated a rearrangement of *HMGA2* [9].

Overall, the test seems to be well suited to detect truncated *HMGA2* transcripts caused by cytogenetically visible genomic rearrangements as well as by those undetectable by conventional cytogenetics.

In conclusion, we were able to identify five leiomyomas with a strongly reduced expression of the full-length mRNA, which is the prevailing transcript in the remaining eight leiomyomas, also showing chromosomal rearrangements affecting 12q14~q15. Thus, a loss of *let-7* complementary sites does not account for the overexpression of *HMGA2* in the majority of UL, but seems to amplify the effects of a transcriptional de-regulation of *HMGA2* in a rather small subset of these tumors. Of note, phenotypic effects of a truncation of the *HMGA2* 3' UTR have also been described in a boy with a pericentric inversion of chromosome 12 that truncated *HMGA2* [19].

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III

HMGA2 and the p19^{Arf}-TP53-CDKN1A axis: A delicate balance in the growth of uterine leiomyomas

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RESEARCH ARTICLES

HMGA2 and the p19^{Arf}-TP53-CDKN1A Axis: A Delicate Balance in the Growth of Uterine Leiomyomas

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Pathogenetically, uterine leiomyomas (ULs) can be interpreted as the result of a monoclonal abnormal proliferation of myometrial cells. Oncogene-induced senescence (OIS) is a frequent phenomenon in premalignant lesions that leads to a growth arrest mainly by the activation of two potent growth-inhibitory pathways as represented by p16^{Ink4a} and p19^{Arf}. The relevance of OIS for the development of UL has not been addressed, but HMGA2, encoded by a major target gene of recurrent chromosomal abnormalities in UL, has been implicated in the repression of the *Ink4a/Arf* (*CDKN2A*) locus. This prompted us to examine if HMGA2 contributes to the growth of leiomyomas by repressing this locus. Contrary to the expectations, we were able to show that generally ULs express significantly higher levels of p19^{Arf} mRNA than myometrium and that UL with 12q14~15 rearrangements showed higher expression levels than UL with other cytogenetic aberrations. Furthermore, the finding of a significant correlation between the expressions of *p19^{Arf}* and *CDKN1A* shows that p19^{Arf} triggers senescence rather than apoptosis in UL. Furthermore, the expression levels of *HMGA2*, *p19^{Arf}*, and *CDKN1A* were found to be correlated with the size of the tumors, indicating that an enhanced growth potential is counterbalanced by the p19^{Arf} pathway. Mechanistically, the UL may thus execute a program already present in their cell of origin, where it is activated to protect the genome, for example, in the case of enhanced proliferation. In summary, the results identify the p19^{Arf}-TP53-CDKN1A pathway as a major player in the growth control and genomic stability of uterine fibroids.

INTRODUCTION

Uterine leiomyomas (ULs) are benign smooth muscle tumors with a high prevalence, making them the most frequent gynecological tumors. On the basis of histology and ultrasound studies, it has been estimated that up to 70–80% of women in their reproductive age have one or more leiomyomas (Cramer and Patel, 1990; Baird et al., 2003). Symptomatic leiomyomas are a major public health problem, accounting for ~30% of all hysterectomies in the United States (Sandberg, 2005). Still, relatively little is known about the pathogenesis and etiology of UL. Although numerous hypotheses have been put forward to explain the development of UL (for review, see Sandberg, 2005), their monoclonal origin suggests mutations as the leading cause of abnormal proliferation of the leiomyoma cells because consistent mutations observed in considerable fractions of UL chromosomal aberrations dominate (for review see Ligon and Morton, 2000; Sandberg, 2005). A large subgroup of UL is characterized by clonal translocations affecting chromosomal region 12q14~15, leading to upregulation of *HMGA2* (Schoenmakers et al., 1995; Gross et al., 2003; Klemke et al., 2009). Interestingly, ULs with 12q14~15 rearrangements are larger than those without detectable cytogenetic deviations (Rein et al., 1998; Hennig et al., 1999). Although it is tempting to speculate that the overexpression of HMGA2, a protein abundantly expressed in stem cells and linked to their self-renewal (Li et al., 2006, 2007; Nishino et al., 2008), accounts for that enhanced growth potential, the exact

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mechanisms by which HMGA2 can influence UL growth still remain to be resolved. Recently, a link between HMGA2 and the *CDKN2A* locus (encoding $p16^{Ink4a}$ and $p19^{Arf}$) was reported for somatic stem cells. Expression of that locus is associated with the control of cellular senescence in many cell types and was found to be repressed by HMGA2 (Nishino et al., 2008). Accordingly, it can be speculated that repression of the *CDKN2A* locus by the abundance of HMGA2 accounts for the larger size of UL with *HMGA2* rearrangements compared with those without that mutation (Rein et al., 1998; Hennig et al., 1999). By immunohistochemistry, $p16^{Ink4a}$ positivity was found more often in leiomyosarcomas than in leiomyomas, where it appears to be restricted to single cases only (Atkins et al., 2008; Lee et al., 2009), but in-depth studies addressing the expression of *Ink4a/Arf* and the genes of their corresponding pathways have not been performed in different genetic subtypes of UL.

Herein, we have investigated a series of ULs with 12q14∼15 rearrangements and other aberrations for their expression of $p16^{Ink4a}$ and $p19^{Arf}$.

MATERIALS AND METHODS

Tissue Samples and Cells

Samples of ULs and myometrium were taken during surgery, immediately frozen in liquid nitrogen, and stored at −80°C for RNA isolation. As a reference for the expression of the *CDKN2A* locus, human adipose tissue–derived stem cells (ADSCs) isolated from subcutaneous adipose tissue were used. Subcutaneous adipose tissue was taken during surgery from patients admitted to the Department of General and Vascular Surgery, Clinical Center Bremen-Nord, Bremen, Germany. For cell culture, the tissue was transferred into sterile Hank's solution and minced into small pieces followed by a treatment with 0.26% (200 U/ml) collagenase (Serva, Heidelberg, Germany). After 1–2 hr, the dissociated cells were transferred into sterile 25-cm^2 cell culture flasks containing 5-ml medium 199 supplemented with 20% fetal bovine serum (FBS) (Invitrogen, Karlsruhe, Germany) and antibiotics (2% penicillin–streptomycin; Biochrom, Berlin, Germany). The cultures were incubated in 5% CO_2 air at 37°C, and medium was changed every 2–3 days. Cultures were passaged when reaching 80% confluence using 1× concentrated TrypLE Express in a PBS-EDTA buffer. The cells were subcultured in medium 199 with 10% FBS and antibiotics.

RNA Isolation

Total RNA was isolated from frozen tissue samples and from cell cultures by using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), and DNase I digestion was performed following the manufacturer's instructions.

cDNA Synthesis

Two hundred and fifty nanograms of total RNA was reverse transcribed with M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany), RNase Out (Invitrogen, Karlsruhe, Germany), random hexamers, and dNTPs according to the manufacturer's instructions. RNA was denatured at 65°C for 5 min and subsequently kept on ice for 1 min. After adding the enzyme to the RNA primer mixes, samples were incubated for 10 min at 25°C to allow annealing of the random hexamers. Reverse transcription was performed at 37°C for 50 min followed by inactivation of the reverse transcriptase at 70°C for 15 min.

Quantitative Real-Time PCR

Relative quantification of transcription levels was carried out by real-time PCR analyses using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Relative mRNA levels of *HMGA2*, $p19^{Arf}$, $p16^{Ink4a}$, *CDKN1A*, *MDM2*, and *MKI67* were determined by relative quantification referring to the expression of the housekeeping gene *HPRT*, which turned out to be expressed steadily in our own experiments on leiomyomas and myometrial tissue. For quantification, the assays Hs00924091_m1 ($p19^{Arf}$), Hs00923893_m1 ($p16^{Ink4a}$), Hs00171569_m1 (*HMGA2*), Hs99999142_m1 (*CDKN1A*), Hs01066930_m1 (*MDM2*), and Hs00606991_m1 (*MKI67*) (Applied Biosystems, Darmstadt, Germany) were used. Primers and probe used to amplify *HPRT* were the same as those previously described by Specht et al. (2001). All experiments were done in triplicate.

RESULTS

First, 36 ULs and eight myometrial tissues were tested by qRT-PCR for their expression of $p16^{Ink4a}$ and $p19^{Arf}$ mRNA. Although the control

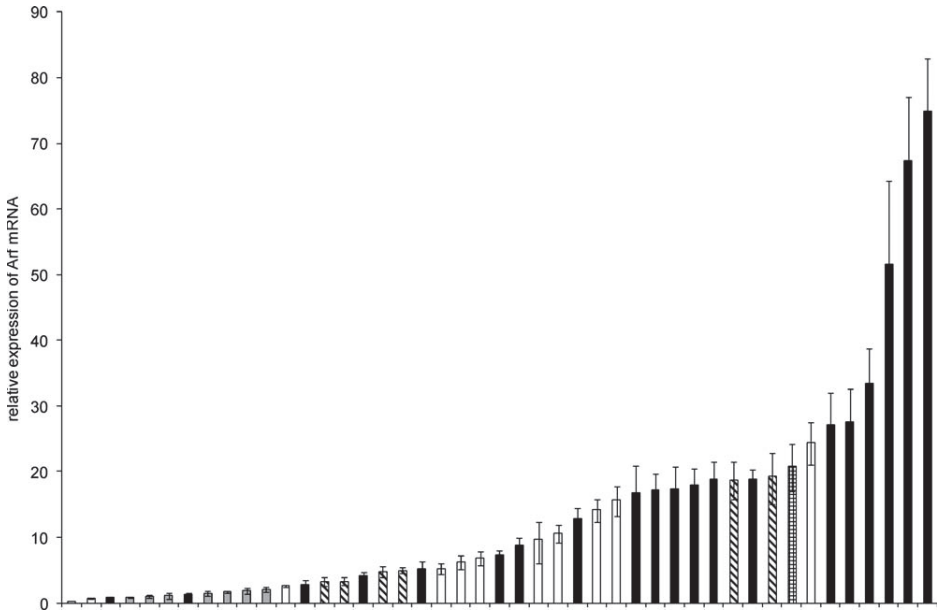

Figure 1. Relative quantification of the *p19*^{Arf} expression in uterine leiomyomas and myometrial tissues. Grey bars: myometrium; white bars: UL with normal karyotype; black bars: UL with 12q14∼15 aberrations; hatched bars: UL with other clonal cytogenetic aberrations, checkered bar: ADSCs (6th passage). Myometrial tissue served as calibrator (expression: 1).

(ADSCs, sixth in vitro passage) but none of the samples showed a detectable expression of p16^{Ink4a} mRNA, p19^{Arf} mRNA was detectable in all samples, in the same range as in the ADSCs (Fig. 1). On the basis of the cytogenetic analyses, the leiomyomas were further divided into a group with 12q14∼15 aberrations ($n = 20$), a group with normal karyotypes ($n = 10$), and a group with other clonal cytogenetic aberrations ($n = 6$). Generally, the expression of *p19*^{Arf} varied over a broad range in UL, whereas it was almost identical in the myometrial tissues. The relative p19^{Arf} mRNA expression in the UL significantly ($P < 0.01$) exceeded that in myometrial tissue by more than 12-fold with an average *p19*^{Arf} mRNA level of 1.303 (range: 0.182–2.119) in myometrial tissue and 16.199 (range: 0.701–74.829) in ULs. Furthermore, in UL with 12q14∼15 aberrations, p19^{Arf} mRNA was expressed at higher levels than in UL with normal karyotypes (21.604 vs. 9.633, $P < 0.05$). Comparing the *p19*^{Arf} expression of UL with 12q14∼15 aberrations with myometrial tissue resulted in a 16.6-fold ($P < 0.01$) higher expression in the aberrant leiomyomas. Because these findings suggest HMGA2 as an agonist of the p19^{Arf}-TP53 pathway, we reasoned that this pathway may be a major player in controlling the growth of UL.

Enhanced levels of TP53 can either induce apoptosis or an irreversible growth arrest, that is, senescence. Although previous data (Dixon et al., 2002) excluded apoptosis as a prominent feature of UL based on the analyses of the apoptosis-regulating proteins BCL2 and BAX, overexpression of β-galactosidase in UL compared with matching myometrium points to a possible significance of senescence. Accordingly, we analyzed if the expression of *CDKN1A*, a direct target of transcriptional regulation by TP53 within the senescence route, is positively correlated with the expression of *p19*^{Arf}. For these analyses, only 19 ULs with 12q14∼15 rearrangements and 10 with an apparently normal karyotype were used. A strongly positive linear correlation between the two mRNAs was noted ($P < 0.001$; Fig. 2). Similar to what was found for *p19*^{Arf}, ULs with 12q14∼15 rearrangements expressed higher ($P < 0.05$) levels of p21 mRNA than those with an apparently normal karyotype. Next, we examined if the expression of *HMGA2*, *p19*^{Arf}, and *CDKN1A* correlates with the size of the UL. For these analyses, data on 24 ULs, including nine with an apparently normal karyotype and 15 with 12q14∼15 rearrangements, were available. For all the three genes, a positive correlation with the size of the UL was noted (Fig. 3). In contrast, none of them showed any correlation with proliferation, as indicated by the expression of MKI67 mRNA (data not shown). Because a positive feedback loop between TP53 and MDM2 is well documented, we

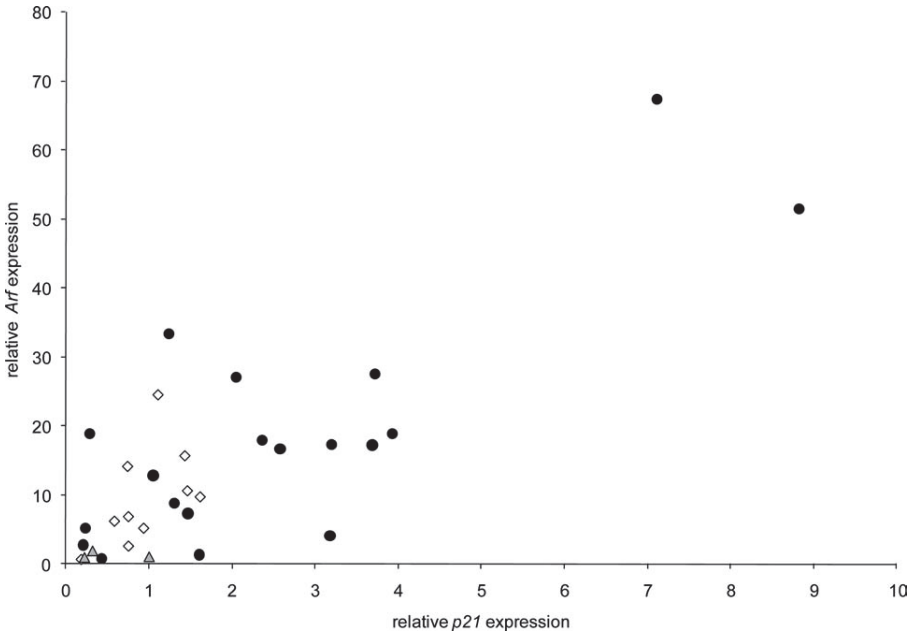

Figure 2. Correlation between the relative *CDKN1A* expression (*x* axis) and the relative $p19^{Arf}$ expression (*y* axis) in myometrium (▲), UL with 12q14∼15 aberrations (●), and UL with a normal karyotype (◇). Myometrial tissue served as calibrator (expression: 1).

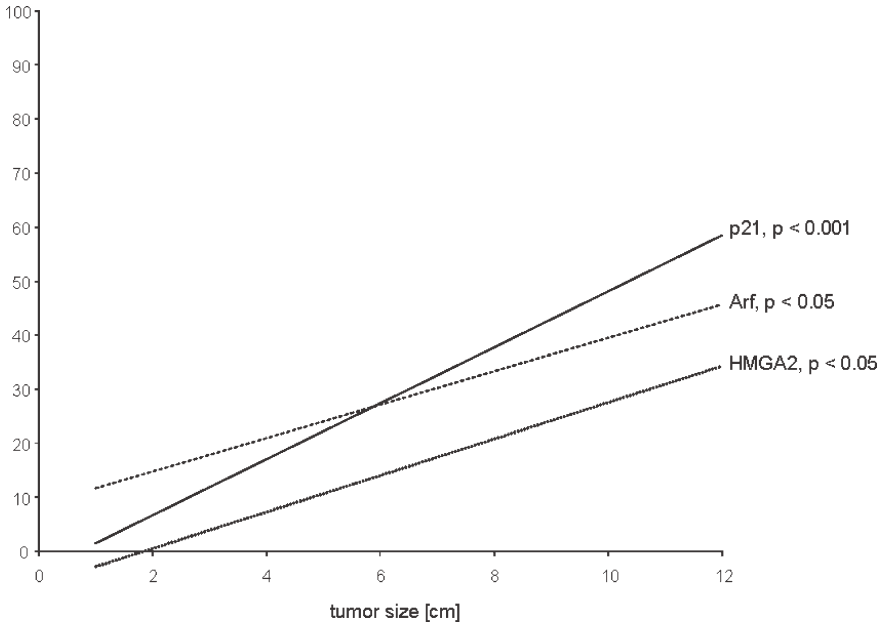

Figure 3. Significant linear correlations between the expression of *HMGA2*, $p19^{Arf}$, and *CDKN1A* (*y* axis, %), respectively, and the size of fibroids investigated. For the expression of all the three genes, the tumor with the highest expression each is adjusted to 100%.

were interested to analyze if enhanced expression of $p19^{Arf}$ triggers an increased expression of *MDM2* as well. A highly significant linear correlation ($P < 0.001$) between the expressions of $p19^{Arf}$ and *MDM2* was noted (Fig. 4). Moreover, fibroids with 12q14∼15 rearrangements expressed significantly ($P < 0.05$) higher levels of MDM2 mRNA than those with an apparently normal karyotype.

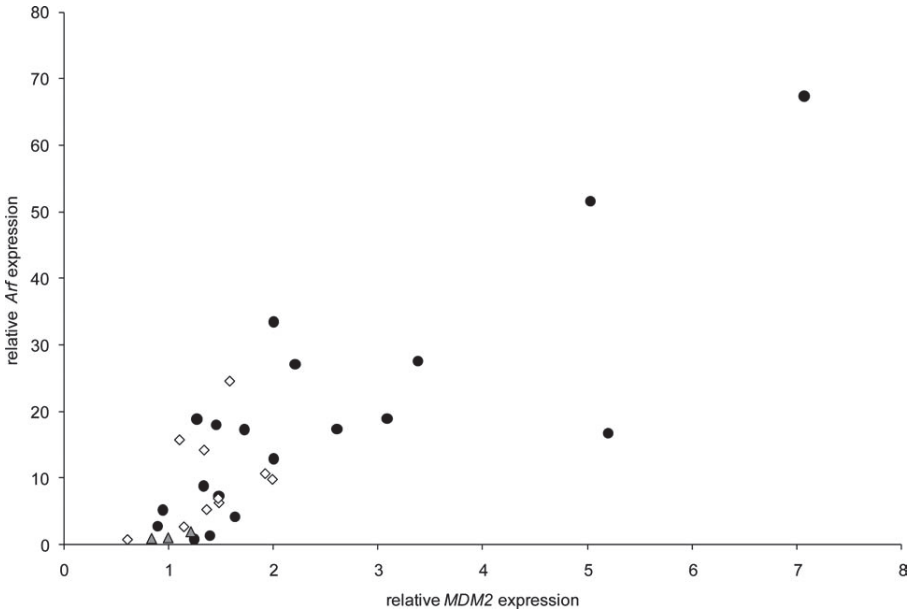

Figure 4. Correlation between the relative *MDM2* expression (*x* axis) and the relative $p19^{Arf}$ expression (*y* axis) in myometrium (▲), UL with 12q14∼15 aberrations (●), and UL with a normal karyotype (◇). Myometrial tissue served as calibrator (expression: 1).

DISCUSSION

ULs are highly frequent benign tumors of women in their reproductive age. In numerous studies, the influence of the hormonal environment as well as, for example, gene polymorphisms, epigenetics, a familial disposition, or even oncogenic viruses (Romagnolo et al., 1996; Webster et al., 1998; Bullerdiek, 1999; Asada et al., 2008) and recently the deregulation of micro-RNA genes (Luo and Chegini, 2008; Marsh et al., 2008; Wei and Soteropoulos, 2008) have been considered as factors related to the development of these tumors. Fibroids are clonally arising from the proliferation of smooth muscle cells (SMCs) or their progenitors. Nevertheless, although cytogenetic analyses allow to distinguish between different cytogenetic subtypes of ULs and point to genes of pathogenetic relevance for the disease, generally, very little is known about downstream mechanisms linked to its pathogenesis. In one of these cytogenetic subtypes, rearrangements of the chromosomal region 12q14∼15 lead to an upregulation of *HMGA2* expression.

Recent data suggesting a relationship between the expression of HMGA2 and the repression of the senescence-associated proteins encoded by the *CDKN2A* locus (Nishino et al., 2008) prompted us to examine if HMGA2 exerts at least in part its stimulation of SMC growth by repression of their senescence program. Contrary to expectations, uterine fibroids generally expressed one gene of the *CDKN2A* locus, that is, $p19^{Arf}$, at significantly higher levels than normal myometrium. In contrast, no such differences were noted for $p16^{Ink4a}$, the other gene of the locus. The two proteins encoded by the *CDKN2A* locus, that is, $p16^{Ink4a}$ and $p19^{Arf}$, are cell cycle inhibitors that both have been linked to cellular senescence. Of these, $p16^{Ink4a}$ blocks phosphorylation and inactivation of the retinoblastoma protein (RB1), whereas $p19^{Arf}$ blocks the ubiquitylation and degradation of TP53 via its interaction with MDM2 (Zhang et al., 1998; Meek, 2009). In primary fibroblasts, $p19^{Arf}$ seems to mediate a network that enforces Ras-induced cell cycle arrest and tumor suppression (Sebastian and Johnson, 2009). Upregulation of $p19^{Arf}$ is part of oncogene-induced senescence (OIS), a term coined to indicate a form of senescence resulting from activated oncogenes and oncogenic viruses. Besides being part of the TP53 network, $p19^{Arf}$ is also known to have TP53-independent antiproliferative activities. Mice lacking $p19^{Arf}$, *MDM2*, and *TP53* in combination develop a much broader spectrum of tumors than animals lacking $p19^{Arf}$ or *TP53* alone. The tumors arise rapidly and can appear simultaneously at independent sites, where they can involve mesenchymal, epithelial,

hematopoietic, or neural cells, suggesting that the $p19^{Arf}$-MDM2-TP53 pathway is not strictly linear (Lowe and Sherr, 2003).

Generally, both proteins of the *CDKN2A* locus have been implicated in the suppression of neoplastic growth, and the *CDKN2A* locus is among the most frequently inactivated gene loci in human cancers (Lowe and Sherr, 2003). Mice carrying a targeted deletion of the *CDKN2A* locus eliminating both $p16^{Iin4a}$ and $p19^{Arf}$ develop spontaneous tumors at an early age and are highly sensitive to carcinogenic treatments (Serrano et al., 1996). In line with these findings, it has recently been shown that cells with low endogenous $p19^{Arf}$ levels and immortal fibroblasts deficient in components of the $p19^{Arf}$-TP53 pathway yield induced pluripotent stem cell colonies with up to threefold faster kinetics and at a significantly higher efficiency than wild-type cells (Utikal et al., 2009).

Our results show that in UL, a stimulation of the $p19^{Arf}$ axis rather leads to senescence than to apoptosis as revealed by an overexpression of *CDKN1A*.

However, the balance between proliferation and senescence seems to be in a delicate balance, and the correlation between $p19^{Arf}$ and MDM2 suggests an oscillation between both gene activities, which has been described to result from a positive TP53-MDM2 feedback loop (Proctor and Gray, 2008; Jolma et al., 2010). As to the development of UL, the higher expression of $p19^{Arf}$ may thus account for the unexpectedly lower inherent ex vivo growth potential of UL cells compared with myometrial cells (Carney et al., 2002; Loy et al., 2005; Chang et al., 2010) as well as to the higher rate of β-galactosidase-positive cells (Dixon et al., 2002). Simultaneously, Chang et al. (2010) were also able to demonstrate that UL exhibited fewer stem and progenitor cell characteristics, respectively, than matching myometrium. Generally, the presence of a stem cell–like population in the myometrium has been postulated and, for example, been linked to changes of uterine smooth muscle tissue during postpartum involution (Shynlova et al., 2009). Accordingly, a study by Ono et al. (2007) revealed the existence of a stem cell–like side population of quiescent multipotent human myometrial cells, which they referred to as myoSP cells. On the basis of these findings, they have suggested that repeated menstruation-induced hypoxia may cause clonal proliferation of a myoSP cell that would ultimately result in the development of a UL, which as a secondary event can acquire cytogenetic abnormalities. This hypothesis traces back the origin of ULs to initially multipotent stem cells and is in line with the multilineage differentiation occasionally seen in UL, that is, lipoleiomyoma or chondroleiomyoma.

However, the highly significant upregulation of $p19^{Arf}$ and *CDKN1A* in UL offers prima facie supporting data explaining their lower in vitro growth potential compared with normal tissue and does neither exclude a stem cell origin of UL nor contradict the loss of stem cell characteristics. In addition, the fact that fewer stem cell colonies are formed from tissue taken from UL than from myometrium (Chang et al., 2010) may be due to advanced senescence and fits with the correlation between $p19^{Arf}$ and CDKN1A mRNA expression found in this study.

In Figure 5, available data on the relevant pathway are summarized. The cell of UL origin may be a myoSP cell or a more differentiated cell where an unknown oncogenic event is supposed to trigger a mild form of OIS leading to an upregulation of $p19^{Arf}$ compatible with slow clonal proliferation. In the subset of UL with chromosomal rearrangements of 12q14∼15, the drastically upregulated HMGA2 expression can induce or strengthen the OIS in a dose-dependent manner. Second, the expression of $p19^{Arf}$ and the corresponding pathway increases with the growth of the tumor cell population as reflected by the correlation between $p19^{Arf}$ and CDKN1A mRNA with tumor size. Overall, the induction of this pathway as well as of the positive feedback loop involving MDM2 may be a reminiscence of the stem cell origin of uterine fibroids and may help the tumor cells to maintain their genomic integrity despite high levels of HMGA2. Finally, we feel that the interplay between HMGA2 and $p19^{Arf}$/CDKN1A, which apparently, among other factors, depends on the degree of overexpression of HMGA2, seems to be one of the key elements determining the final size of UL. Interestingly, OIS was also recently considered as a cause for the spontaneous cessation of growth of pituitary adenomas (Mooi, 2009), benign endocrine tumors that share with leiomyomas the frequent upregulation of HMGA2 (Fedele and Fusco, in press).

In summary, OIS governed by the $p19^{Arf}$-p21 axis seems to be an important phenomenon in the development of UL and cessation of their growth. HMGA2, encoded by a gene targeted in a frequent genetic subtype of UL, and the senescence-associated $p19^{Arf}$ and p21 are assumed to

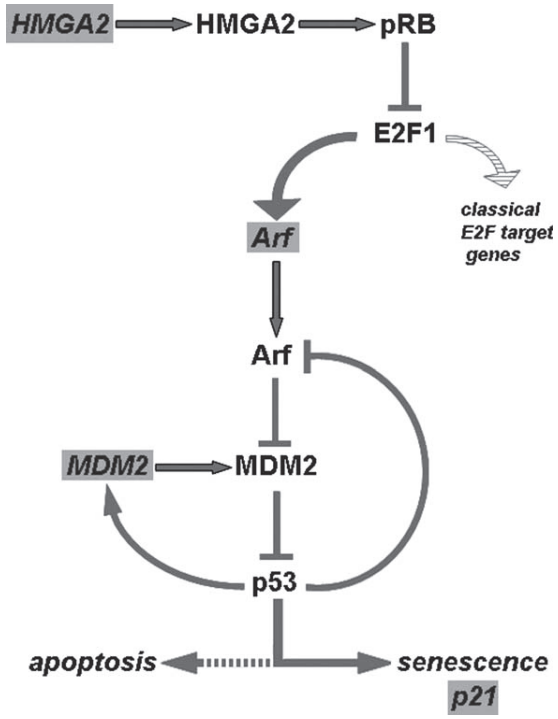

Figure 5. Model delineating the interaction of key elements of a senescence pathway active in uterine leiomyomas. The model is based on the finding that leiomyomas express significantly higher levels of $p19^{Arf}$ mRNA than myometrial tissue. Most likely due to the repression of MDM2 followed by the stabilization of TP53 this corresponds to an increase of *CDKN1A* expression, the latter being a direct target of transcriptional activation by TP53. In turn, a negative feedback loop between TP53 and $p19^{Arf}$ (Robertson and Jones, 1998) and a positive feedback loop between TP53 and MDM2 (Zhang et al., 1998; Meek, 2009) exist. The higher expression of $p19^{Arf}$ in UL with 12q14∼15 rearrangements compared to those of other cytogenetic subtypes suggests that *HMGA2* may behave like a classical oncogene inducing $p19^{Arf}$-driven oncogene induced senescence. Though it is not clear how HMGA2 can influence $p19^{Arf}$ transcription one hypothetical way might be via its possible interaction with pRB1 (Fedele et al., 2006) followed by the activation of $p19^{Arf}$ by E2F1 (cf. Komori et al., 2005). Of note, other mechanisms of direct or indirect interaction between HMGA2 and the *CDKN2A* locus e.g. at the chromatin level are likely to exist and other so far unknown factors may be able to induce that pathway in fibroids as well. However, this pathway may keep fibroids in a delicate balance between growth and senescence and ensure high genomic stability despite high levels of HMGA2. Genes investigated in the present study are highlighted by grey background and alternative routes of possible minor relevance in fibroids are represented by hatched lines.

be the major players interacting in different ways to dictate the fate of an individual UL.

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IV

6p21 rearrangements in uterine leiomyomas targeting *HMGA1*

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- Conventional cytogenetics
- Gene expression analyses
- Drafting the manuscript
- Final approval of the manuscript

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6p21 rearrangements in uterine leiomyomas targeting *HMGA1*

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Abstract To quantify the expression of *HMGA1* mRNA in uterine leiomyomas, the expression of *HMGA1* was analyzed in a series including tumors with aberrations of chromosome 6 ($n = 7$) and cytogenetically normal tumors ($n = 8$) as a control group by quantitative reverse transcriptase–polymerase chain reaction. The average expression level in the 6p21 group was found to be 5.6 times higher than that in the control group, and with one exception, all cases with 6p21 alteration revealed a high expression of *HMGA1* mRNA than cytogenetically normal tumors. Nevertheless, compared to fibroids with a normal karyotype, the upregulation of the *HMGA1* mRNA in these cases was much less strong than that of *HMGA2* mRNA in case of 12q14~15 aberrations identified in previous studies.

1. Introduction

Uterine leiomyomas (ULs) belong to the cytogenetically best investigated human tumors. The cytogenetic analyses have revealed several subtypes, with a frequent group showing rearrangements of chromosomal region 12q14~15, which apparently targets the gene encoding the high-mobility group AT-hook 2 (*HMGA2)* [1,2]. Accordingly, tumors of this type show significantly higher expression of *HMGA2* than fibroids with an apparently normal karyotype [3]. HMGA2 is a protein abundantly expressed in stem cells and casually linked to their self-renewal ability. A decrease of HMGA2 has recently seen linked to the group of hematopoietic as well as neural stem cells [4]. Accordingly, it is tempting to speculate that in terms of pathogenesis, smooth muscle cells continuously expressing HMGA2 are maintaining a self-renewing program that occasionally also display multilineage potential as witnessed by variants as, for example, lipoleiomyomas or leiomyomas with cartilaginous differentiation [5,6].

Of note, a smaller subgroup of ULs shows rearrangements of 6p21 (i.e., the locus where *HMGA1,* the other gene encoding proteins of the HMGA type, has been mapped), suggesting that *HMGA1* is the relevant target gene in that subgroup of ULs [7]. In small series of ULs, it was shown that this rearrangement leads to an overexpression of *HMGA1* [8,9]. However, to our knowledge, no study quantifying the expression of *HMGA1* mRNA in ULs of this subtype has been performed. Thus, we analyzed the *HMGA1* expression in seven ULs with aberrations of chromosome 6 in comparison to myomas with normal karyotype and to the matching myometrial tissues.

2. Materials and methods

2.1. Tissue samples and chromosome analysis

For RNA isolation, samples of ULs and myometrium were snap frozen in liquid nitrogen immediately after surgery and stored at −80°C. For cell culture, samples of primary tumors were transferred to Hank's solution with antibiotics (200 IU/mL penicillin, 200 μg/mL streptomycin) after surgery. Cell culture and chromosome analyses were performed as described previously [3].

2.2. RNA isolation, reverse transcription, and quantitative reverse transcriptase–polymerase chain reaction

Total RNA was isolated from tissue samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany) including

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DNase 1 treatment according to the manufacturer's instructions, and quantitated by spectrophotometry. Reverse transcription of 250 ng RNA was carried out with M-MLV reverse transcriptase, RNaseOUT, and random hexamers (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Controls without reverse transcriptase were included for each sample to ensure the absence of DNA contaminations, which, as a result of the high number of *HMGA1*-related retropseudogenes, could lead to false-positive results.

Quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed on a real-time

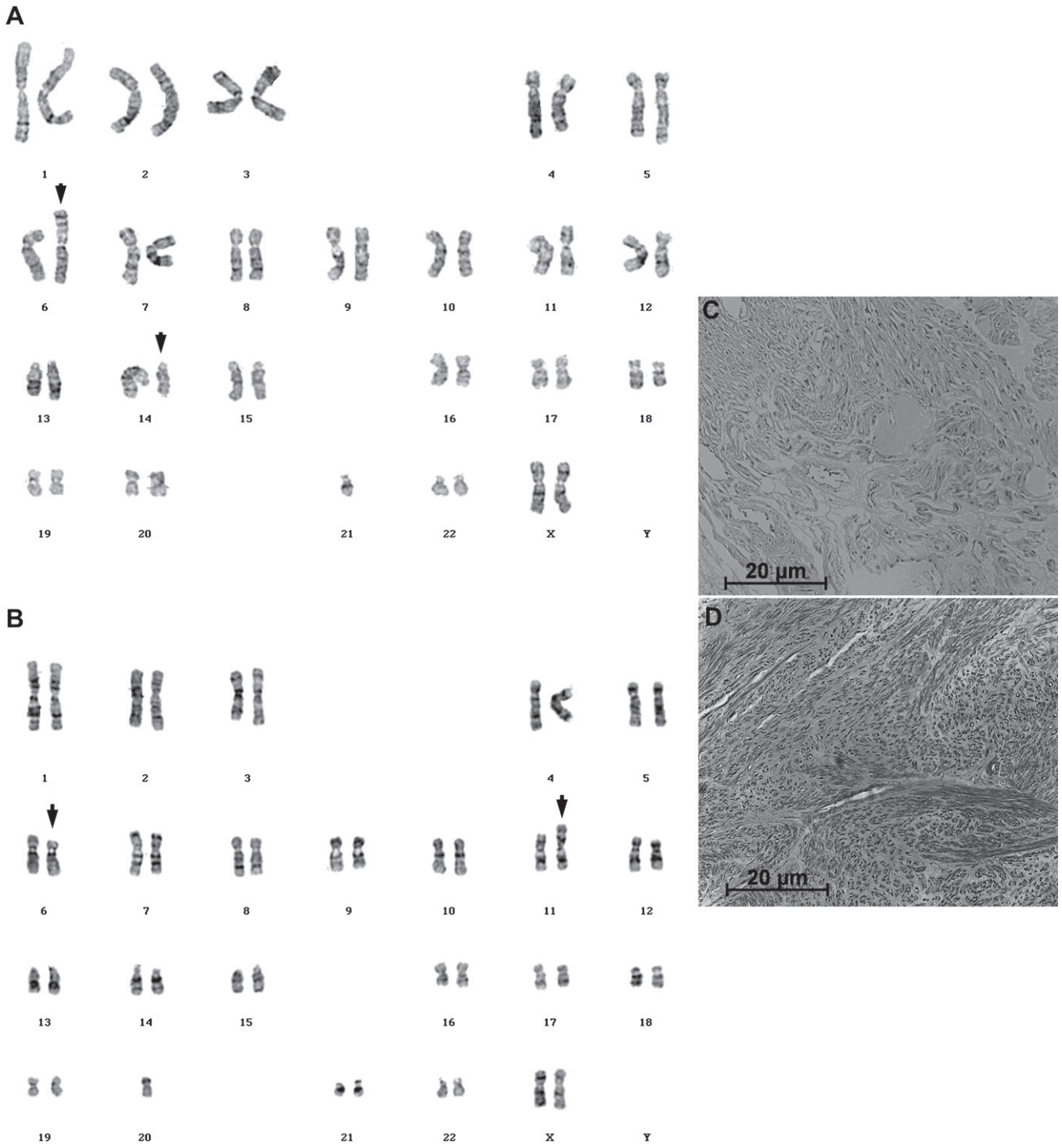

Fig. 1. Karyotypes of two ULs with 6p21 rearrangements with different levels of *HMGA1* expression and the histologic appearance of these ULs. (A) Representative G-banded karyotype of myoma 87: 46,XX,t(6;14)(p23;q24), tas(14;21)(pter;qter). (B) Representative G-banded karyotype of myoma 125A: 46,XX, t(6;11)(p21;p15), chromosomes participating in the 6p21 rearrangements are indicated by arrows. Histologic appearance of myoma 87 (C) and myoma 125A (D).

PCR cycler (Applied Biosystems, Darmstadt, Germany) with TaqMan Universal Mastermix. Of each cDNA, 2 μL served as template in a final reaction volume of 20 μL. Reaction condition were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C. For transcripts of *HMGA1,* a set of primers and probe was designed (forward primer: 5′-GGA CCA AAG GGA AGC AAA AA-3′, reverse primer: 5′-TTC CTG GAG TTG TGG TGG TTT-3′, probe: 6-FAM-AAG GGT GCT GCC AAG ACC CGG-MGB). 18S rRNA was chosen as endogenous control and detected with the following primer/probe set: forward primer: 5′-GGA TCC ATT GGA GGG CAA AGT-3′, reverse primer: 5′-AAT ATA CGC TAT TGG AGC TGG AAT TAC-3′, probe: TGC CAG CAG CCG C [10]. All reactions were run in triplicate.

2.3. Analysis of gene expression

The relative expression was calculated by the ΔC_t method, using 18S rRNA as endogenous control and choosing the *HMGA1* expression of a myometrial sample (of normal group) as calibrator. For statistical analyses, Student's *t*-test was used. *P*-values of ≤0.05 were considered to be significant.

2.4. Fluorescence in situ hybridization

For determination of rearrangements involving 6p21 and *HMGA1,* respectively, fluorescence in situ hybridization (FISH) was performed on metaphase preparations of the cases myoma 36, myoma 110, myoma 121B, myoma 125A, and myoma 166A. For FISH, two overlapping clones CTD-2522J1 (GenBank accession number AQ280064 and AQ280066) and CTD-2510D13 (GenBank accession number AQ264849 and AQ264850), both located distal to *HMGA1* in 6p21, and two overlapping clones CTD-2524P4 (GenBank accession number AQ310763 and AQ277896) and RP11-140K17 (GenBank accession number AQ385566 and AQ385568), both located proximal to *HMGA1,* in 6p21 were used. CTD clones were obtained from Invitrogen (Darmstadt, Germany); RP11-140K17 was obtained from imaGenes (Berlin, Germany). DNA was isolated with the Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany).

One microgram of isolated plasmid DNA was labeled by nick translation (Abbott Molecular, Wiesbaden, Germany) either with SpectrumOrange-dUTP (CTD-2522J1 and CTD-2510D13) or SpectrumGreen-dUTP (CTD-2524P4 and RP11-140K17) (Abbott Molecular). Treatment of metaphases and subsequent FISH experiments were carried out as described previously [11]. Twenty microliters of the

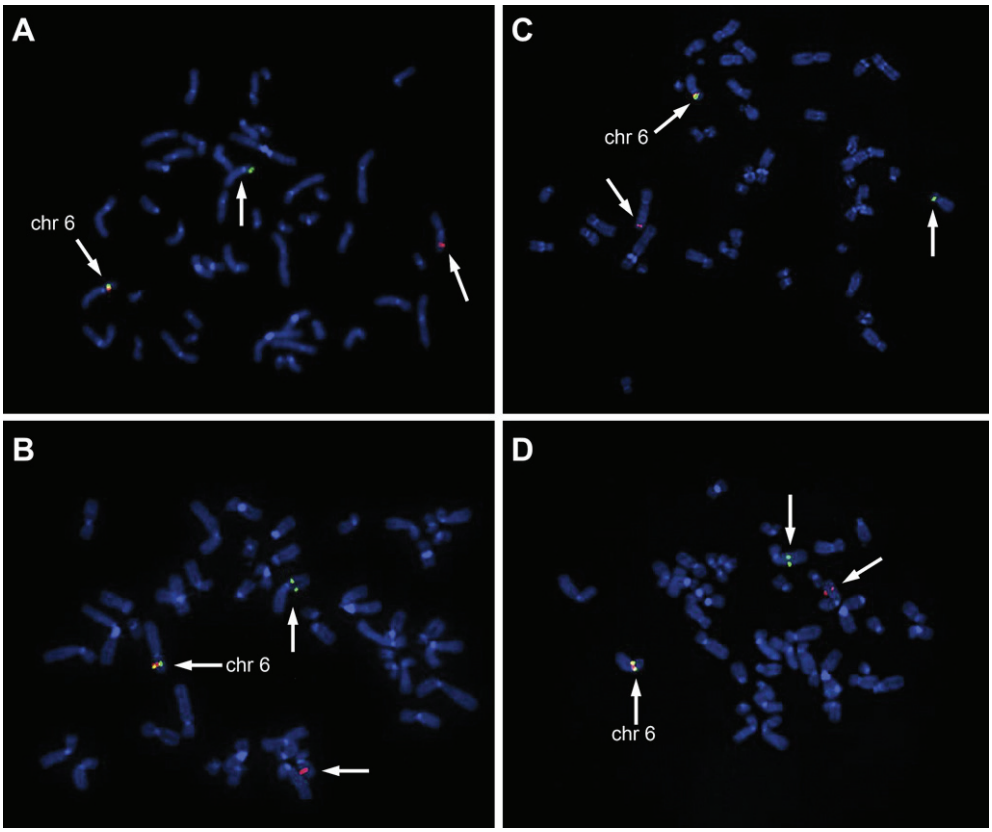

Fig. 2. FISH analysis with *HMGA1* break-apart probes in four UL with 6p21 rearrangement. (A) Myoma 125A, (B) myoma 166A, (C) myoma 121B, (D) myoma 110 (for karyotypes see Table 1). The green fluorescent probes (CTD-2524P4 and RP11-140K17) located proximal to *HMGA1*, the red fluorescent probes (CTD-2522J1 and CTD-2510D13) located distal to *HMGA1*. Arrows point to normal chromosomes 6 and the rearranged chromosomes.

Table 1
Cytogenetic and molecular-cytogenetic data, results of relative *HMGA1* and *HMGA2* expression, and clinical data of all ULs. All tumors investigated were histologically typical leiomyomas.

Case no.	Karyotype	Relative *HMGA1* expression	Relative *HMGA2* expression	Patient age (y)	Tumor size (cm)	*HMGA1* expression matching myometrium	Single or multiple	FISH analysis for *HMGA1* rearrangements
87	46,XX,t(6;14)(p23;q24)[6]/46,XX,t(6;14)(p23;q24),tas(14;21)(p13;q22)[11]/46,XX[2]/47,XX,+12[1]	0.5	0.6	63	—	1.7	Multiple	ND
125A	46,XX,t(6;11)(p21;p15)[7]/46,XX[14]	32.0	2.7	42	2.5	2.3	Multiple	Split signals
110	44,XX,der(1)t(1;?),der(3),der(5)t(5;?),−6,der(11)?t(11;15)(q25;q22),del(15)(q22),der(15)t(15;?),−19[25]	33.2	1.6	44	4	NA	Single	Split signals
113C	46,XX,t(6;10)(p23;q23)[5]/46,XX[7]	34.0	11.4	53	3.5	NA	Multiple	ND
117D	46,XX,t(6;11)(p23;q21)[4]/46,XX[12]	41.2	26.0	39	4.5	NA	Multiple	ND
121B	42~46,X,−X[6],−1[19],t(1;8)(p22;q24)[11],der(1)[6],del(3)[3],add(6)[19],−8[6],der(8)[4],−10[6],−11[6],−13[6],−14[19],−22[15],+mar1[18],+mar2[18],+mar3[6],+mar[6][cp19]/46,XX[1]	63.3	0.8	38	3	NA	Multiple	Split signals
166A	46,XX,t(6;10)(p21;q22)[13]/46,XX[8]	110.4	1.29	—	3	NA	Multiple	Split signals
14B	46,XX[10]	2.8	3.8	46	—	8.5	Multiple	ND
12B	46,XX[10]	3.1	2.4	41	—	1.0	Multiple	ND
24	46,XX[20]	3.9	0.5	44	1	7.6	Multiple	ND
27A	46,XX[10]	4.8	2.8	40	5	NA	Multiple	ND
20	46,XX[10]	5.1	0.6	36	—	1.2	Multiple	ND
37D	46,XX[15]	10.0	12.9	43	2	15.4	Multiple	ND
29	46,XX[21]	12.6	1.1	48	3.5	8.5	Multiple	ND
36	46,XX[14]	23.2	10.7	39	6	6.9	Single	No split signals

Abbreviations: −, unknown; ND, not done; NA, not available.

break-apart probe was used per slide. Co-denaturation was performed on a ThermoBrite (Abbott Molecular) for 7 minutes at 77°C, followed by overnight hybridization in a humidified chamber at 37°C. Posthybridization was performed at 70°C for 2 minutes in 0.4× standard saline citrate/0.3% NP-40. Metaphases were counterstained with DAPI (4′,6-diamidino-2-phenylindole; 0.75 μg/mL). Slides were examined with an Axioskop 2 Plus fluorescence microscope (Carl Zeiss, Göttingen, Germany). Images were captured with a high-performance CCD camera (Visitron Systems, Puchheim, Germany) and were edited with FISH View (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Chromosomes were identified by inverted DAPI staining. For each case, if possible, at least 10 metaphases were analyzed, and 100 interphase nuclei were scored. Co-localized signals (green/red) indicate a nonrearranged breakpoint region, whereas separated green and orange signals indicate a rearrangement of the chromosomal region 6p21 and *HMGA1,* respectively.

3. Results

In this study, seven ULs with chromosomal rearrangements of 6p21 and eight karyotypically normal ULs as detected by chromosome analysis of GTG-banded metaphases and FISH analysis were subjected to real-time RT-PCR analysis. According to the histological examination, no sign of leiomyosarcoma or atypical leiomyoma was detected in any of the cases (Fig. 1).

For identification of 6p21 rearrangements involving *HMGA1* FISH on metaphase preparations and interphase nuclei of five myomas (four cases with 6p rearrangement or loss of one normal chromosome 6, respectively, and one with apparently normal karyotype) was performed with a *HMGA1*-specific break-apart probe. The results revealed a signal pattern corresponding to a 6p21 rearrangement involving *HMGA1* in all cases except for that with a normal karyotype (numbers 110, 121B, 125A, and 166A; Fig. 2A–D).

The expression of *HMGA1* mRNA in seven ULs with 6p21 rearrangement was compared to that in eight samples of UL with an apparently normal karyotype, which served as controls (Table 1). For nine of these samples, matching myometrium was available. Two myometrial samples belonged to fibroids of the 6p group, and the remaining tissues belonged to fibroids of the normal group. The average relative expression level in the 6p group was 45-fold compared to its expression in a myometrium sample of normal group as calibrator and differed significantly from that in the control group (8.2-fold increase). As to the expression in the individual tumors, *HMGA1* expression, with one exception, clearly distinguishes between both karyotypic groups (Fig. 3). In the exception, case 87, no unusually high percentage of metaphases with normal karyotype could be detected, which may explain the low expression of *HMGA1* observed. Regarding the expression of *HMGA1* in UL and matching myometrium, there were no significant differences in the normal group.

Next, we analyzed whether the expression of *HMGA1* correlates with the expression of *HMGA2*. No evidence for such a correlation was obtained (Fig. 3).

4. Discussion

ULs are by far the most common gynecological tumors, occurring in at least 70–80% of all women in their reproductive years [12–14]. Cytogenetic subtypes have been identified that may correlate with a different molecular pathogenesis of the disease. So far, the best-investigated group is characterized by 12q14~15 changes associated with a strong overexpression of *HMGA2* [3,15]. The

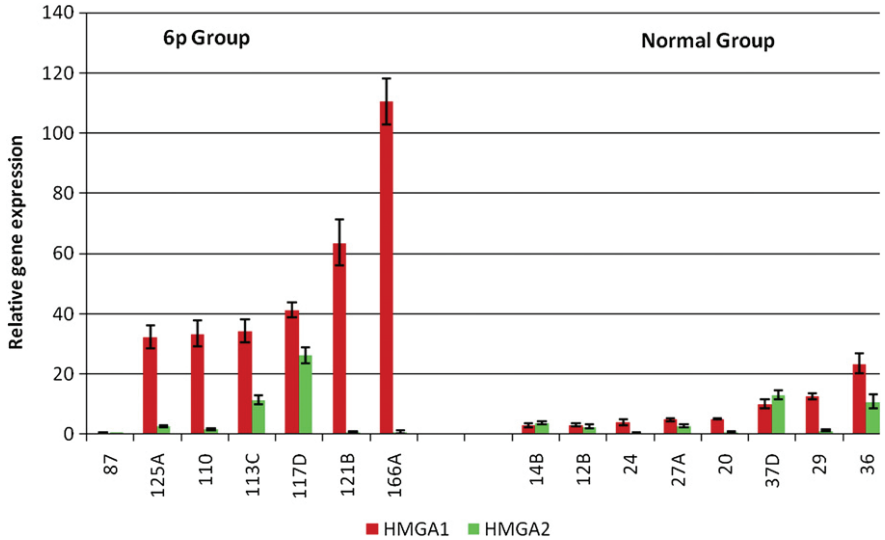

Fig. 3. Relative quantification of the *HMGA1* and *HMGA2* expression in normal and aberrant uterine leiomyomas (ULs).

overexpression of the stem-cell chromatin-associated protein HMGA2 fits well with important characteristics of UL growth. A much smaller subset of UL is characterized by 6p21 rearrangements leading to the upregulation of a closely related protein of the HMGA family (i.e., *HMGA1*). Likely activation of either of both genes and the abundance of their proteins, respectively, leads to an almost identical histopathologic phenotype of the tumors commonly referred as leiomyomas.

However, what distinguishes tumors of both types is the level of overexpression of *HMGA* genes compared to myometrium. Whereas the average upregulation of *HMGA2* in case of 12q14~15 aberrations is in range of 3,000-fold [3], even when omitting the outlier represented by case 87, upregulation of *HMGA1* due to 6p21 rearrangements is on average 45-fold and raise up to a maximum of only 52.4-fold.

We had recently been able to show that most cytogenetically normal leiomyomas show subtle changes of the *HMGA2* level compared to the matching myometrium as well [3]. Some recent studies have correlated HMGA2 with stemness of mesenchymal cells and stem cell self-renewal [4,16]. Although similar studies are lacking for HMGA1, it is tempting to assume that the translocation products of both genes can shift mesenchymal stem cells or progenitors of smooth muscle cells and other mesenchyme-derived cells back to a higher self-renewing potential. Nevertheless, we were recently able to show that there is no correlation between the expression of *Ki-67* and *HMGA2* [17]. Thus, the exact mechanism by which increased levels of HMGA proteins contribute to benign tumorigenesis still remains to be elucidated. However, HMGA1 can be assumed to have the ability to replace, at least in part, the function of HMGA2 and vice versa. The proteins of both genes are highly charged DNA-binding proteins that show abundant expression in embryonic cells but greatly decreased expression in most adult cells. They share a high homology in their DNA-interacting domains; despite their apparent differences in interaction partners [18], this may explain why knockout for neither gene alone is lethal.

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V

12q14~15 aberrations do not enhance the chromosomal instability in uterine fibroids

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Submitted

Own contribution:

Conventional cytogenetics

Drafting the manuscript

12q14~15 Aberrations do not Enhance the Chromosomal Instability in Uterine Fibroids

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The stem cell chromatin associated protein HMGA2 is discussed as a mutagenic protein inducing genomic instability. Nevertheless, it is unlikely that HMGA2 which is abundantly expressed during embryonic and fetal life impairs the integrity and stability of genome. We have addressed this apparent contradiction by considering the increased number of metaphases with single cell aberrations as a hallmark of genomic instability. From the results, there was no evidence for an increased genomic instability in the group of uterine leiomyomas with 12q14~15 rearrangements leading to *HMGA2* overexpression compared with the group of normal myomas.

Recently, some evidence has been presented (Li et al., 2009) that the chromatin associated high mobility AT-hook 2 (HMGA2) can act as a mutagenic protein inducing DNA-double strand breaks as well as polyploidy. However, the findings have been challenged because in embryonic stem cells and a couple of highly frequent benign tumors as e.g. leiomyomas, lipomas, and pulmonary chondroid hamartomas an abundant expression of *HMGA2* is not accompanied by a marked genomic instability or an increased tendency of malignant transformation (Sandberg, 2005; Morton, 1998). Thus, it has been proposed that HMGA2 may contribute to genomic instability only very high concentrations. However, to the best of our knowledge it has not been investigated as yet if in benign tumors an over expression of *HMGA2* correlates with increased genomic instability as reflected by an increased number of metaphases with single cell aberrations. One type of tumor offering good chances for this type of investigations are uterine leiomyomas (UL). Whereas the majority of UL display an apparently normal karyotype. UL with clonal aberrations targeting the

HMGA2-locus (Ashar et al., 1995; Schoenmakers et al., 1995) leading to its overexpression (Tallini et al., 2000; Gross et al., 2003; Klemke et al., 2009) are a frequently occurring subtype. Herein we have compared the frequency of single cell aberrations in 20 UL with 12q14~15 aberrations (Fig.1: (a) and (b)) with that of 184 UL with an apparently normal karyotype (Fig.1: (d)). All tumors included in this study were additionally analyzed by qRT-PCR for their expression of HMGA2 mRNA in order to detect hidden *HMGA2* rearrangements (Fig.2). Within the 12q14~15 group of UL the average *HMGA2* expression was found to be 102-fold higher than in the group with a normal karyotype. Nevertheless, there was no evidence for an increased genomic instability in the former group (Tab. 1) nor was there any evidence for an increase rate of polyploid cells.

It seems plausible to assume that the cells having 12q14~15 abnormalities protect the genome despite their high HMGA2. Narita and colleagues mentioned that the HMGA proteins also act in tumor suppressor networks by having a role in cellular senescence and heterochromatin formation (Narita et al., 2006). The study by Markowski et al., (2010) revealed a high expression of senescence-associated $p19^{Arf}$ in the presence of overexpression of *HMGA2* in UL with 12q14~15 rearrangements. Results of this study identify the $p19^{Arf}$-TP53-CDKN1A pathway as a balancer in the growth and genomic stability of UL in presence of a high level of HMGA2. Therefore, the role of tumor suppressor protein p53 in senescence and genetic stability should be attended. The data by Izadpanah et al. (2008) presented that there is a coincidence between the arrest in the S phase of the cell cycle, detected in the long-term *in vitro* culture of human mesenchymal stem cells (MSCs) and the considerably suppressed expression of p53. Therefore, seems 12q14~15 aberrations leading to an overexpression of HMGA2 do not increase the instability of genome in uterine leiomyomas.

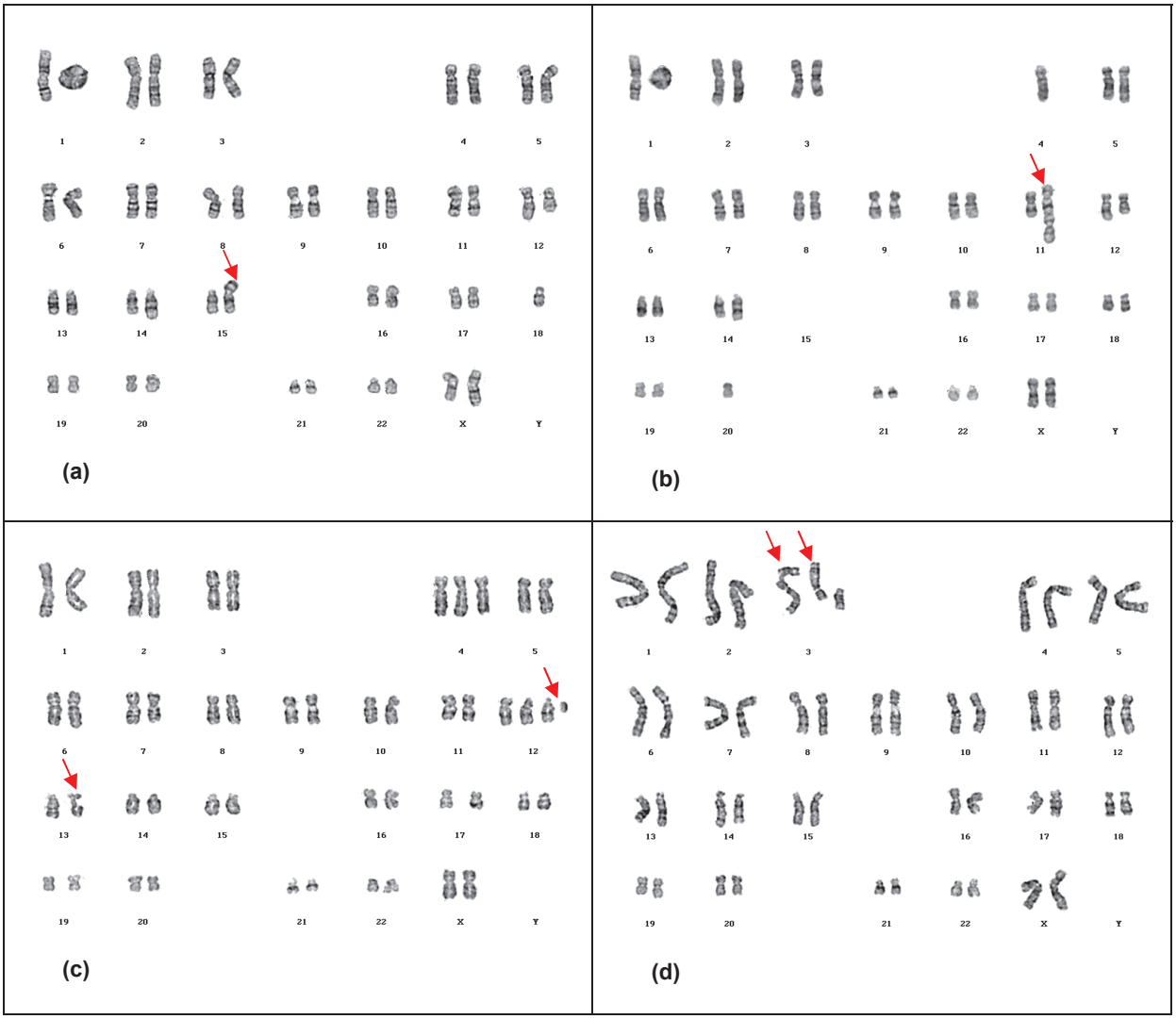

Fig.1: Karyograms of myomas including single cell aberration or chrb/chtb.
(a) Myoma 612: 45,XX,r(1),t(12;14),der(15)t(15;18),-18; (b) Myoma 612: 42,XX, der(1)r(1;?),-4,dic(11;?;15), t(12;14) (q15;q24),-15,-15,-20; (c) Myoma 551.2: 48,XX,+4,+12, chrb(12)(p), chtb(13)(q); (d) Myoma 590.1: 46,XX, Chrb(3)(q), Chtb(3)(p).

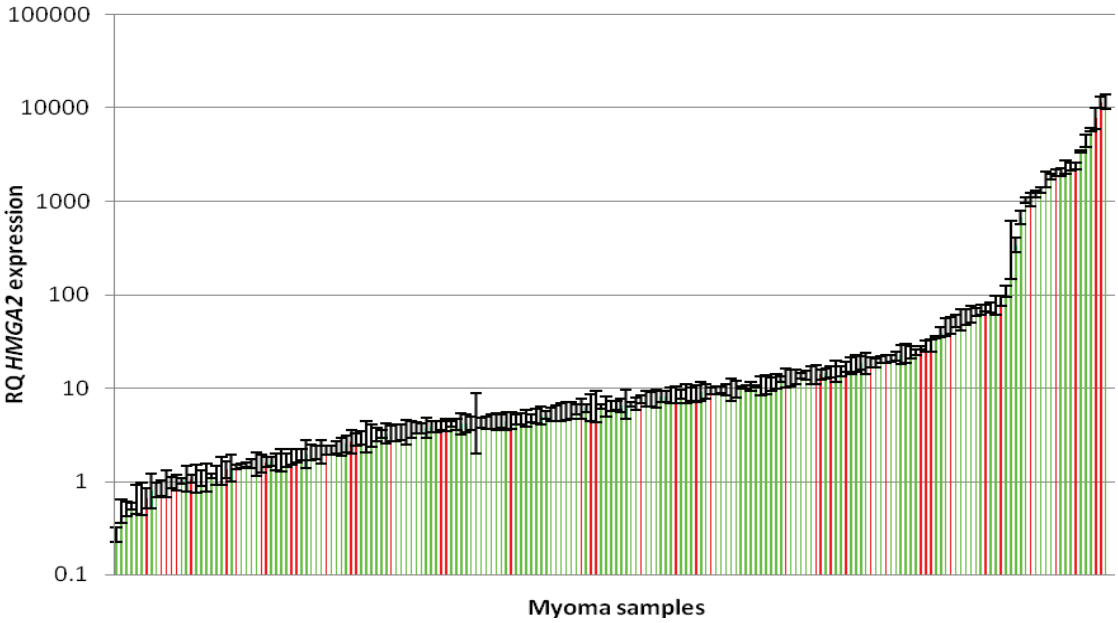

Fig. 2: RQ *HMGA2* expression in leiomyomas.
Green bars: UL without single aberration or chrb/chtb; red bars: UL with single aberration or chrb/chtb.

Tab. 1: Results of the analysis of the metaphases in different cytogenetic subgroups.

Cytogenetic group	All affected myomas with single aberration or chrb/chtb	12q aberrant myomas without single aberration or chrb/chtb	Normal myomas without single aberration or chrb/chtb	All 12q aberrant myomas	All normal myomas
Total samples	46	16	151	20	184
Range of analysed metaphases	6-29	6-27	5-27	6-27	5-29
Total analysed metaphases	740	270	1915	341	2431
Average of analysed metaphases	16	17	13	17	13
Total karyotyped metaphases	402	144	930	180	1205
Average karyotyped metaphases	9	9	6	9	7
Total affected samples	46	0	0	4	33
Percentage of Total affected samples	100%	0%	0%	20%	17.9%
Total affected metaphases	63	0	0	6	45
Percentage of Total affected metaphases	8.5%	0%	0%	1.76%	1.85%

Abbreviations: chrb/chtb: chromosome break/chromatid break.

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