Untersuchungen zur Genetik von Speicheldrüsentumoren

[Genetic Analyses of Salivary Gland Tumors]

DISSERTATION

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"The more you look, the more you find!"

Robert A. Weinberg

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Summary

Salivary gland tumors are a morphological very heterogeneous group of tumors. This morphological diversity in combination with the relative rareness, especially of the salivary gland carcinomas makes these tumors to a major challenge for pathologists, also for those specialized to these tumors. In the recent years molecular biomarkers become increasingly important, in order to facilitate the work of the pathologist and the treating clinician.

Aim of this work was to analyze the recently for mucoepidermoid carcinoma (MEC) and Warthin's tumor (WAT) described gene fusion *CRTC1-MAML2* for its diagnostic potential as biomarker and to find out if an aberrant *HMGA2* expression level influence tumorgenesis of MECs. For this thesis we have tested approximately 140 MECs and 50 WATs for the *CRTC1-MAML2* fusion and checked for correlations between fusion status and patient data. Furthermore, we have analyzed approximately 60 MECs for their *HMGA2*-expression level, by real-time PCR.

Our studies have shown that a *CRTC1-MAML2* test could be a powerful tool for diagnosis and prognosis of MECs. The fusion correlates with a low- or intermediate-grade of the tumor and is associated with a favorable prognosis. Interestingly, *CRTC1-MAML2* negative tumors were mainly found in high-grade tumors and show a significant increased *HMGA2* level and a poor prognosis. These data raise doubts about the correct classification of MECs into low-, intermediate- and high-grade tumors. In our opinion the present classification of MECs includes two subgroups: A huge group of “true-MECs” (ca. 70% of the tumors) with the *CRTC1-MAML2* fusion, with a moderate aggressiveness and an excellent prognosis and furthermore a smaller group of morphological heterogeneous high-grade tumors lacking this fusion and with an aberrantly high *HMGA2* expression level. We suggest that this “non-MEC” group comprised not only one specific tumor type.In fact it seems to be a mix of different tumor types, all poorly differentiated with typical high-grade features. Based on these studies, we approve that all MECs should be analyzed for the *CRTC1-MAML2* fusion in the future routinely. In particular in border cases (intermediate vs. high-grade) the test may be very useful to do correct diagnosis.

Our study shows that the *CRTC1-MAML2* fusion seems to be a rare event in WATs. These fusion positive tumors are indicated for a potential malignant transformation. In

this field further studies are recommended. Nevertheless, we give advice to test WAT for the presence of *CRTC1-MAML2* and monitoring patients with this fusion more closely; analogous to follow-up practices for other salivary gland adenomas at risk for recurrence or progression diseases.

Key Words:

Mucoepidermoid carcinoma, Warthin's tumor, *CRTC1-MAML2*, *HMGA2*, molecular biomarker, prognostic significance

Zusammenfassung

Speicheldrüsentumoren sind eine Gruppe von morphologisch sehr vielfältigen Tumoren. Diese morphologische Vielfalt in Kombination mit der relativen Seltenheit, vor allem der Karzinome der Speicheldrüse stellen besondere Herausforderungen für den Pathologen da. Oft stoßen hier auch auf Speicheldrüsentumoren spezialisierte Pathologen an ihre Grenzen. Molekulare Biomarker gewannen daher in den letzten Jahren immer mehr an Bedeutung, um die Arbeit des Pathologen und des behandelnden Arztes zu erleichtern.

Ziel dieser Arbeit war es das kürzlich beschriebene Fusionsgen *CRTC1-MAML2* in Mukoepidermoidkarzinomen (MEC) und Warthin-Tumoren (WAT) näher auf ihr diagnostisches Potential als molekularer Biomarker zu untersuchen. Des Weiteren sollte eine mögliche Beteiligung des Onkogen *HMGA2* auf die Tumorgenese von MEC mittels Real-Time PCR untersucht werden. Insgesamt wurden im Rahmen dieser vorliegenden Arbeit rund 140 MEC und 50 WAT auf die *CRTC1-MAML2* Fusion hin untersucht und sofern möglich mit den Patientendaten auf mögliche Korrelationen abgeglichen. Rund 60 MEC wurden darüberhinaus noch auf ihre *HMGA2*-Expression untersucht.

Unsere Ergebnisse weisen auf ein großes Potential der *CRTC1-MAML2* Fusion für die Diagnostik und Prognostik hin. Das Auftreten der Fusion korreliert mit einem niedrigem Tumorgrad und einer exzellenten Prognose. Interessanterweise zeigen unsere Untersuchungen, dass das Nichtvorhandensein der Fusion für eine schlechte Prognose und einer zum Teil deutlich erhöhten *HMGA2*-Expression in High-Grade Tumoren steht. Dies legt den Verdacht nahe, dass die aktuelle Klassifizierung der MEC in Low-, Intermediate- und High-Grade Tumoren einer Überarbeitung bedarf. Die Derzeitige Gruppe der MEC scheint in Subgruppen aufgeteilt zu sein: In eine große Gruppe von „echten-MEC“ (ca. 70% der Tumore) mit *CRTC1-MAML2*-Expression und einer exzellenter Prognose, welche sich aus Low- und Intermediate-Grade Tumore zusammensetzt und in eine heterogene Gruppe von „nicht-MEC“ Tumoren, welche die High-Grade Tumore umfasst und durch die Abwesenheit von *CRTC1-MAML2* und einer zum Teil deutlich erhöhten *HMGA2*-Expression gekennzeichnet ist. Der zweiten Gruppe könnten einer ganzen Reihe von unterschiedlichen Karzinomen mit typischer High-Grade Struktur angehören. Wir denken daher, dass ein Test auf *CRTC1-MAML2* vor allem bei der korrekten

Einstufung von Grenzfällen (Intermediate- versus High-Tumor), sehr hilfreich sein kann und Routinemäßig angewandt werden sollte.
Unsere Untersuchungen an WAT zeigen, dass die *CRTC1-MAML2* Fusion auch hier auftritt, allerdings nur sehr selten und möglicherweise hier einen Hinweis auf eine maligne Transformation des Tumors gibt. In diesem Bereich sind noch weitere Studien notwendig. Wir Empfehlen dennoch auch WAT in Zukunft Routinemäßig auf *CRTC1-MAML2* zu testen und positive Fälle einer genauen Folgebeobachtung zu unterziehen; analog zu Adenomen der Speicheldrüse die dazu neigen können rezidive auszubilden, oder zu Folgeerkrankungen führen können.

Schlüsselwörter:
Mukoepidermoidkarzinom, Warthin-Tumor, *CRTC1-MAML2*, *HMGA2*, molekularer Biomarker, prognostische Signifikanz

List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Fehr A*, Röser K*, Belge G, Löning T, Bullerdiek J.** 2008a. A closer look at Warthin tumors and the t(11;19). Cancer Genet Cytogenet 180(2):135-9.

II. **Fehr A, Röser K, Heidorn K, Hallas C, Löning T, Bullerdiek J.** 2008b. A new type of MAML2 fusion in mucoepidermoid carcinoma. Genes Chromosomes Cancer 47(3):203-6.

III. **Verdorfer I, Fehr A, Bullerdiek J, Scholz N, Brunner A, Krugmann J, Hager M, Haufe H, Mikuz G, Scholtz A.** 2009. Chromosomal imbalances, 11q21 rearrangement and MECT1-MAML2 fusion transcript in mucoepidermoid carcinomas of the salivary gland. Oncol Rep 22(2):305-11.

IV. **Fehr A, Meyer A, Heidorn K, Röser K, Löning T, Bullerdiek J.** 2009. A link between the expression of the stem cell marker *HMGA2*, grading, and the fusion *CRTC1-MAML2* in mucoepidermoid carcinoma. Genes Chromosomes Cancer 48(9):777-85.

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Introduction

Salivary Gland Tumors

Salivary glands are exocrine organs responsible for the production and secretion of saliva. They comprise three paired major glands (parotid, submandibular and sublingual) and numerous minor glands. The minor glands are widely distributed throughout the mouth and oropharynx.

Salivary gland tumors are relatively rare neoplasms accounting for between 3% and 10% of head and neck tumors in the literature. The estimated global incidence rate ranges from 0.4 to 13.5 cases per 100.000 annually (Ellis et al., 1991; Pinkston and Cole 1999; Speight and Barrett 2002). Salivary gland neoplasms comprise less than 3% of all tumors in the major glands. Most are benign and the parotid gland is the most common site with between 64 and 80% of all primary epithelial salivary gland tumors. Between 7-11% of benign tumors occur in the submandibular glands, 9-23% in the minor glands and less than 1% occurs in the sublingual glands (Eneroth 1971; Eveson and Cawson 1985a; Spiro 1986; Ellis et al., 1991). As a general rule, the smaller the involved salivary gland, the higher the possibility of the tumor being malignant. So the percentage rates for malignant salivary gland tumors are approximate: 25% in parotid glands, 53% in submandibular glands, 77% in sublingual glands and 65% in other minor salivary glands (Eneroth 1971; Eveson and Cawson 1985a; Eveson and Cawson 1985b; Spiro 1986; Speight and Barrett 2002).

Among all patients the most frequent benign tumor type is the pleomorphic adenoma (PA), with a total rate of 55%, followed by Warthin’s tumor (WAT) (alias cystadenolymphoma). Off the malignant tumors the most common are mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (AdCC) (Eneroth 1971; Batsakis et al., 1978; Batsakis and Regezi 1978; Batsakis et al., 1979; Eveson and Cawson 1985a; Spiro 1986; Pinkston and Cole 1999).

The entire diversity of salivary gland tumors is beyond the scope of this thesis. The following chapters should be considered an overview of the most frequent groups of tumors that are focused in this thesis.

Pleomorphic Adenoma

Pleomorphic adenomas (PA) are the most common type of salivary gland tumor and are also known as "mixed tumors". They are slow-growing, usually benign tumors

originating from the minor and major salivary glands, occuring most often in patients between 40 and 50 years of age, and found more commonly in females than in males (Eveson and Cawson 1985a; Spiro 1986; Waldron 1991). The tumors are characterized by architectural pleomorphisms and are composed of epithelial elements intermingled with mucoid, myxoid, or chondroid tissues; they are tumors of encapsulation (Webb and Eveson 2001; Barnes et al., 2005; Zbären and Stauffer 2007). These characteristic features usually allow a correct diagnosis. However, the characteristic heterogeneity of the morphological patterns may also cause problems during diagnosis, particularly in small biopsies. The capsule thickness may vary and is sometimes difficult to detect, especially in PAs with mainly mucous parts (Webb and Eveson 2001) and areas of PA may resemble or be identical to a range of other tumor types including low-grade adenocarcinoma, adenoid cystic carcinoma, basal cell adenoma and epithelial-myoepithelial carcinoma. In addition, PAs may contain areas or show metaplastic changes which resemble other tumor types (Speight and Barrett 2002).

Pleomorphic adenomas are cytogenetically well-characterized tumors. Chromosomal studies of more than 500 tumors have been reported in the literature, and have shown that in addition to cases with an apparently normal karyotype, two frequent groups of cytogenetic abnormalities exist. The first subgroup is characterized by structural aberrations involving chromosomal band 8q12 and the second by alterations of the chromosomal region 12q14-15 (Mark et al., 1980; Stenman and Mark 1983; Bullerdiek et al., 1987b; Bullerdiek et al., 1987c; Sandros et al., 1990; Bullerdiek et al., 1993). There is also a third subgroup which represents tumors with non-recurrent clonal changes (Mark et al., 1983; Bullerdiek et al., 1987a; Mark et al., 1988; Mark et al., 1997; Persson et al., 2008). The target genes in PAs with chromosome rearrangements involving 8q12 and 12q14-15 are *PLAG1* and *HMGA2*, respectively (Schoenmakers et al., 1995; Geurts et al., 1997; Kas et al., 1997; Geurts et al., 1998; Voz et al., 1998; Astrom et al., 1999).

Kas et al. have previously shown that the recurrent t(3;8)(p21;q12) translocation results in promoter swapping between *PLAG1* and the gene for β-catenin (*CTNNB1*), leading to activation of *PLAG1* expression and reduced expression of *CTNNB1*. PLAG1 is a developmentally regulated zinc finger transcription factor, with expression mainly restricted to fetal tissues (Kas et al., 1997). In contrast, CTNNB1 is a ubiquitously expressed protein that is involved in cell-cell adhesion as well as in the

Wingless/Wnt signaling pathway (Peifer 1997; Willert and Nusse 1998; Moon et al., 2002). In addition to the *CTNNB1-PLAG1* fusion; a few variant fusions have been identified in PA: *FGFR1-, LIFR-*, *CHCHD7-* and *TCEA1-PLAG1*. The fusion *PLAG1-LIFR* results from the recurrent translocation t(5;8)(p13;q12), *CHCHD7-* and *TCEA1-PLAG1* respectively from reccurent cryptic rearrangements in tumors with normal karyotype. In all of these cases, the rearrangements led to promoter swapping or substitutions, leaving an intact ORF of *PLAG1* (Voz et al., 1998; Astrom et al., 1999; Asp et al., 2006; Persson et al., 2008).

HMGA2 is a chromatin-associated non-histone protein that binds through its AT binding motifs to the minor groove of AT-rich DNA strands and act as an architectural transcription factor (Reeves and Nissen 1990; Bustin and Reeves 1996; Zhou and Chada 1998). The *HMGA2* gene is expressed predominantly during embryogenesis and is normally repressed in differentiated cells and tissues (Chiappetta et al., 1996; Rogalla et al., 1996; Hirning-Folz et al., 1998). *HMGA2* has been shown to be the target gene on chromosome 12 in a variety of benign mesenchymal tumors with rearrangements of 12q13–15, including lipomas, uterine leiomyomas, hamartomas of the breast and lung, fibroadenomas of the breast, angiomyxomas, endometrial polyps, and bone and soft tissue chondromas (Zaidi et al., 2006). Oncogenes containing *HMGA2* often result in an overexpression of *HMGA2.* In malignant neoplasms, a strong association between the overexpression of *HMGA2* and the malignant phenotype and an adverse prognosis has been demonstrated for a variety of malignancies (Rogalla et al., 1997; Rommel et al., 1997; Rogalla et al., 1998; Sezer et al., 2000; Gross et al., 2003; Langelotz et al., 2003; Miyazawa et al., 2004; Sarhadi et al., 2006; Meyer et al., 2007a; Meyer et al., 2007b; Winkler et al., 2007; Belge et al., 2008). An overexpression is also described for benign tumors such as tumors of the salivary glands (Schoenmakers et al., 1995; Kazmierczak et al., 1996). In PAs, three fusion partner genes of *HMGA2* have been identified, *WIF1*, *FHIT* and *NFIB* (Geurts et al., 1997; Geurts et al., 1998; Persson et al., 2009).

Warthin's Tumor (Cystadenolymphoma)

Warthin's tumor (WAT) is a neoplsam composed of glandular and often cystic structures, sometimes with a papillary cystic arrangement, lined by a characteristic bilayered epithelium, comprising inner columnar eosinophilic or oncocytic cells surrounded by smaller basal cells. The stroma contains a variable amount of

lymphoid tissue with germinal centers. Most WATs are well circumscribed and partly cystic. The cysts vary from small bubbles to quite large spaces and contain clear, mucous, creamy white or brown fluid. In the metaplastic variant, solid areas are predominant and often firm and fibrous. The immunoprofile of the lymphocyte subsets is similar to that in normal or reactive lymph nodes. The typical WAT is usually unmistakable in differential diagnosis (Chaudhry and Gorlin 1958; Seifert et al., 1980; Eveson and Cawson 1986; Ellis et al., 1991; Simpson and Eveson 2005). However, in cases of marked regressive changes and subsequent squamous and mucous metaplasia (so-called metaplastic variants), the differential diagnosis of MEC invariably occurs (Seifert et al., 1980; Eveson and Cawson 1986; Di Palma et al., 1999; Schwerer et al., 2001). Much less is known about the cytogenetics of WATs. The majority of tumors analyzed to date have apparently had normal karyotypes (Bullerdiek et al., 1988; Mark et al., 1989,1990; Nordkvist et al., 1994; Martins et al., 1997). The only recurrent abnormality detected was a t(11;19)(q21–22;p13) which was first described by Bullerdiek et al. (1988). This translocation is of particular interest because an identical rearrangement has also been identified in MEC, where it has been found to be one of the most frequent cytogenetic aberrations (Behboudi et al., 2006; Okabe et al., 2006; Fehr et al., 2008b). Recent studies have shown that this translocation results in the same *CRTC1-MAML2* gene fusion in WAT and MEC (see next chapter) (Tonon et al., 2003; Enlund et al., 2004; Martins et al., 2004; Tirado et al., 2007; Fehr et al., 2008a). However, the number of WATs analyzed to date is limited, so it is premature to draw conclusions about the overall pattern of abnormalities in these tumors.

Mucoepidermoid Carcinoma

Mucoepidermoid carcinoma (MEC) is the most common malignant tumor of the salivary glands and was first reported in 1945 (Stewart et al., 1945). The tumor is characterized by squamoid (epidermoid), mucous-producing and intermediate-type cells. The proportion of different cells types and their architectural configuration varies within and between tumors. The histological picture is characterized by prominent cysts in addition to cellular structures, and at times the tumor is associated with a lymphatic proliferation. For the differential diagnosis these variances of the MEC architecture are very problematical because the single cell types could also occur in other tumor types. This complicates a correct diagnosis and the prognosis of

the tumor (Ellis and Auclair 1996; Goode and El Naggar 2005; Luna 2006). In rare cases MECs evolve in benign salivary gland tumors, like carcinoma ex pleomorphic adenoma (CaPA), WAT or oncocytoma (Gnepp et al., 1989).

Since the 1950s, several grading systems have been proposed to grade this neoplasm, but none has been universally accepted (Foote Jr and Frazell 1953; Batsakis and Regezi 1978; Spiro et al., 1978; Evans 1984; Auclair et al., 1992; Brandwein et al., 2001). However, one recent system based on a score system using five histopathologic features has been shown to be reproducible, this system consistently differentiates among three grades: low- (with good prognosis), intermediate- and high-grade (with poor prognosis) tumors (Goode and El Naggar 2005; Luna 2006).

The translocation t(11;19)(q21;p13) has been the most frequent cytogenetic aberration in MEC with a frequency of about 38%-81% (Martins et al., 2004; Behboudi et al., 2006; Okabe et al., 2006; Tirado et al., 2007; Fehr et al., 2008b). Recent cloning of the translocation in MEC cell lines identified a fusion gene comprised of exon 1 of the *CREB regulated transcriptional coactivator CRTC1* (also known as *MECT1*, *TORC1* or *WAMTP1*) gene on chromosomal band 19p13 and exon 2-5 of the Mastermind-like gene *MAML2* on chromosomal band 11q21 (Tonon et al., 2003; Enlund et al., 2004). The fusion protein is expressed in all MEC-specific cell types (Behboudi et al., 2006). Previous functional studies have shown that the expression of *CRTC1-MAML2* is essential for the growth of t(11;19) positive MEC cell lines and that the N-terminal cAMP response element-binding (CREB) binding domain is fundamental for the transforming activity of the chimeric protein (Coxon et al., 2005; Wu et al., 2005; Komiya et al., 2006). Similar to *Drosophila Mastermind* and *MAML1*, full-length *MAML2* acts as a CSL-dependent transcriptional co-activator of Notch (Wu et al., 2000; Lin et al., 2002; Wu et al., 2002). In contrast, CRTC1-MAML2 activates the transcription of Notch target genes independent of Notch ligands and CSL binding sites. In addition, the recent identification of the CRTC1 protein as a potent co-activator for genes that are regulated by cAMP-responsive elements suggests that CRTC1-MAML2 may act by the disruption of both Notch and CREB-regulated cell-cycle and differentiation pathways and induce tumorigenesis (Conkright et al., 2003; Iourgenko et al., 2003; Wu et al., 2005).

Diagnosis and Prognosis of Salivary Gland Tumors

Salivary gland tumors are a morphologically and clinically diverse group of neoplasms, which may present considerable diagnostic and management challenges to the pathologist and surgeon. They are challenging for two main reasons. First, the complex morphologies of salivary gland tumors often overlap among different histological tumor types and the total number of different tumor types which must be distinguished in the differential diagnosis has been increased in the last years (Thackray et al., 1972; Seifert et al., 1991; Barnes et al., 2005). Second, the histological characteristics of these tumors also present challenges. Clinical practice shows that the clinical behavior of some salivary gland tumors does not correlate very well with the tumor classification. Tumors of the same tumor class may have different clinical characteristics. This shows the importance of a good classification but also the importance of suitable molecular tools for diagnosis and prognosis.
In the recent years there have been advances in histology, but there are only a few efficient molecular genetic and immunohistochemical biomarkers for the practical diagnosis of carcinoma and their functionality is limited (Caselitz et al., 1983; Matsuba et al., 1986; Childers et al., 1996; Simpson et al., 2003; Leivo 2006). New molecular tools are needed for a better prognosis of carcinoma of the salivary glands.

Where can usefule genetic biomarkers be found?

The key to this question could be fusion genes. Recurrent balanced rearrangements have been found in almost every tumor type and many of these changes are explicitly associated with distinct tumor phenotypes, clinical features and gene expression profiles (Mitelman et al., 2009). Recurrent balanced rearrangements are considered important early events during tumorigenesis. Chromosomal translocations are the most commonly encountered balanced rearrangements and they commonly results in tumor-specific fusion oncogenes. Two mayor types of oncogenes have been identified: The first type is the deregulation of a gene through exchange of regulatory elements (e.g. promoter swapping), and the second is fusions that often result in the creation of a fusion gene that comprises the coding regions of two different genes (Aman 2005; Stenman 2005; Mitelman et al., 2007).

Aims of the Thesis

In this thesis we investigated the diagnostic and prognostic value of the *CRTC1-MAML2* gene fusion and of the *HMGA2* expression level in MEC, in consideration of the clinical data.

In detail the main aims of the papers included in this thesis were the following:

Paper I – To analyze how widespread the *CRTC1-MAML2* fusion is in WAT.

Paper II – To study the role of the *CRTC1-MAML2* fusion in MECs. Is there a correlation between the tumor grade and the fusion status? Are alternative fusion partner involved?

Paper III – To determine genetic alterations in MECs of the salivary glands in association with clinical and histopathological parameters in a small cohort of MECs with follw-up data.

Paper IV – To investigate if there a relationship between the *CRTC1-MAML2* fusion status, the tumor grade and the *HMGA2* expression level in MEC.

Materials and Methods

For detailed descriptions of the materials and methods, see the individual papers (I-IV), or the references therein. Supplemental information is provided here.

Tumor Material and Cell Lines

Tumor material was donated from the follow institutions:

Albertinen Pathologie	Hamburg, Germany
Gerhard-Domagk-Institut für Pathologie	Muenster, Germany
Institut für Anatomie II, UKE	Hamburg, Germany
Institut für Hämatopathologie	Hamburg, Germany
Institut für Pathologie, Medizinische Universität Innsbruck	Innsbruck, Austria
Speicheldrüsenregister der Oral Pathologie, UKE	Hamburg, Germany

More than two hundred benign and malign salivary gland tumors and a few tumors from other sites were examined. The tumors were mainly archived FFPE material. Most of the examined WAT and the control salivary gland tissues were fresh frozen material. All tumors were carefully examined by at least two pathologists in accordance with the criteria of the WHO (Barnes et al., 2005). Chromosome abnormalities were described according to the International System for Cytogenetic Nomenclature ISCN (2005).

As positive control for the expression of the fusiontranscript *CRTC1-MAML2* we used the well-described NCI-H292 cell line for the studies.(Yoakum et al., 1983; Banks-Schlegel et al., 1985; Carney et al., 1985; Tonon et al., 2004).

Buffers, Solutions and Culture Media

Unless otherwise specified, all buffers, solutions and culture media were made following the instructions of Sambrock et al. (1989).

Kits

High Pure RNA Paraffin Kit	Roche
pGEM-T Easy Vector System I	Promega
QIAEX II Gel Extraction Kit	QIAGEN
QIAGEN DNeasy Blood & Tissue Kit	QIAGEN
QIAGEN Plasmid Midi Kit	QIAGEN
QIAGEN Plasmid Mini Kit	QIAGEN
QIAprep Spin Miniprep Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN
RNeasy FFPE Kit	QIAGEN
RNeasy Mini Kit	QIAGEN
Zyto*Light* MEC I Probe	ZytoVision

cDNA-Synthesis and PCR

cDNA was synthesized from total RNA and mRNA using a poly-T (MACDS, 5'-TTCTAGAATTCAGCGGCCGC$(T)_{20}$VN-3') or gene-specific primer, 5 µg total RNA or 500 ng mRNA, and 200 U M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA from RNA isolated from FFPE material was synthesized with 5 µg total RNA, random hexamer primer, 200 U Superscript™ RT Reverse Transcriptase and 40 U RNaseOut (both Invitrogen) according to the manufacturer's instructions. cDNA quality was checked with *GAPDH*-PCR as described (Fehr et al., 2008a; Fehr et al., 2009).

DNA fragments were amplified with PCR using gene-specific primers following the Taq DNA Polymerase (recombinant) (Invitrogen) or Go Taq Flexi DNA Polymerase (Promega) protocols. The annealing temperatures were varied and minor modifications made depending on the primers (for details see the respective publications). The PCR reaction took place in a Mastercycler Gradient (Eppendorf) in 0.2 ml Eppendorf PCR reaction tubes. To avoid carry-over contamination we used uracil-DNA glycosylase and dUTPs instead of dTTPs in most PCR reactions in the dNTP-mix as described (Longo et al., 1990).

Sequencing of PCR-Products

Samples were sequenced by the companies GATC Biotech AG and Eurofins MWG Operon, or as described by Fehr et al. (2008b).

In Silico Analysis

The genomic DNA contigs, cDNA contigs, protein predictions, and the homology alignments were created with Lasergene (Version 3 and 5, DNAStar) or Vector NTI software (Version 10, Invitrogen).

Results

CRTC1-MAML2 Fusion in WAT (Paper I)

As previous described, *CRTC1-MAML2* is not exclusively expressed in MEC, it is also expressed in WATs, but till yet only a few cases have been tested for this fusion. To find out how widespread the *CRTC1-MAML2* fusion might be in WATs we carried out nested RT-PCR on forty-eight WATs. This revealed the expression of the chimeric gene in two metaplastic WATs, but in none of the forty-six remaining ordinary WAT-cases. On review, the two positive cases were classified as tumors that were probably MECs (Fehr et al., 2008a).

CRTC1-MAML2 and *HMGA2* in MEC (Papers II, III and IV)

To gain a better understanding of the role of *CRTC1-MAML2* in MEC we tested 138 FFPE MECs for the fusion transcript. Approximate 70% of the examined cases showed evidence of an expression of the *CRTC1-MAML2* fusion gene (Fehr et al., 2008b; Fehr et al., 2009; Verdorfer et al., 2009). The fusion transcript was mainly found in low-grade and intermediate-grade tumors; only a few *CRTC1-MAML2* positive samples were high-grade tumors, on the other hand *CRTC1-MAML2* negative tumors were in most cases high-grade tumors (Fehr et al., 2008b; Fehr et al., 2009; Verdorfer et al., 2009). Additionally, a highly differentiated MEC revealed an aberrant amplification product. Sequencing analysis demonstrated a fusion of *MAML2* with the *CRTC3* gene instead of *CRTC1.* The reading frame of the two genes is not affected by the translocation and a functional protein may be obtained from the sequence (Fehr et al., 2008b).

Nineteen cases were also tested by fluorescence *in situ* hybridization (FISH) on interphase nuclei; a *MAML2*-rearrangement was detected in 37% of the analyzed tumors. These cases were also analyzed with comparative genomic hybridization (CGH). The CGH analysis showed an recurrent over representation of chromosome X and losses of entire chromosomes or regions on chromosomes 1, 2 and 15 as the most frequent copy number changes (Verdorfer et al., 2009).

Fifty-three of the MECs were also tested for their *HMGA2* expression level. In all samples an increased expression of *HMGA2* was detected. The highest expression was found in fusion transcript negative high-grade tumors. In the total cohort of MECs the mean *HMGA2* expression value rose with the tumor grade and was higher in *CRTC1*-MAML2-negative than in fusion-positive tumors. Furthermore, one-way ANOVA revealed a significant difference between the *HMGA2* expression medians of the three grading stages. In summary, the *HMGA2* expression is significantly higher in *CRTC1-MAML2* negative than in positive tumors and increased with the stage of grading (Fehr et al., 2009).

Discussion

Salivary gland tumors display one of the greatest diversities of histology among human cancers. A broad morphologic spectrum exists among different tumor types and sometimes even within an individual tumor mass. In addition, the occurrence of hybrid tumors, dedifferentiation and the propensity of some benign salivary gland tumors to progress to malignancy make this group of lesions one of the most interesting and challenging in the head and neck. Treatment of salivary gland tumors is challenging because of their rarity and their unpredictable biologic behavior. Malignant neoplasms are marked by frequent locoregional failure and distant metastasis, often occurring years or decades after diagnosis (Spiro 1986; Terhaard et al., 2004).

The total number of different tumor types which must be distinguished in differential diagnosis has been considerably increased in the last years, as the existing WHO classifications show (Thackray et al., 1972; Seifert et al., 1991; Barnes et al., 2005). These extensive classifications allow accurate diagnosis so that lesions can be correctly categorized. Finally, because of the rareness of salivary gland tumors it is very difficult for a normal pathologist to find the correct diagnosis, so salivary gland tumors belongs to the hands from specialized pathologists. However, it is by studying subsequent clinical behavior that prognostic groupings of individual diagnostic entities can be established (Speight and Barrett 2002).

The goal of molecular biological studies on salivary gland tumors is to define objective molecular biomarkers that may supplant the subjective phenotypic evaluation in the diagnosis, biological assessment and therapeutic stratification of patients with these tumors. Chromosome aberrations are a characteristic feature of neoplasia, in September 2009 approximate 56.700 cases with chromosome changes across all main cancer types have been reported in “The Mitelman Database of Chromosome Aberrations in Cancer” (Mitelman et al., 2009). Many of these changes are explicitly associated with distinct tumor phenotypes, clinical features and gene expression profiles and there is compelling evidence that they represent an initial event in oncogenesis (Aman 2005; Mitelman et al., 2007). Balanced chromosome abnormalities result in the formation of gene fusions and exert their tumorigenic action by two alternative mechanisms: The first, deregulation of a gene through

exchange of regulatory elements is well documented in hematological malignancies (Adams et al., 1983; Rabbitts 1994). In this type of translocation the promoter region of one gene is fused to the intact coding parts another gene by a process called promoter swapping. One example for promoter swapping in salivary gland tumors is the fusion between *PLAG1* and *CTNNB1* in PAs. The *PLAG1* oncogene is here placed under the control of regulatory elements of the gene for *CTNNB1*, leading to activation of *PLAG1* expression and reduced expression of *CTNNB1* (Kas et al., 1997). The second mechanism of fusion gene formation results in the creation of a chimeric fusion gene that comprises the coding regions of two different genes. The most famous example of a translocation creating a chimeric fusion gene is the Philadelphia chromosome breakpoint with the fusion *BCR-ABL1* (Rabbitts 1994; Look 1997; Deininger et al., 2000; Rowley 2001). Oriented on salivary glands tumors the fusion *CRTC1-MAML2* is an example, for such a mechanism (Tonon et al., 2003). Many recurrent cytogenetic aberrations show a strict specificity for tumor types, *PLAG1* or *HMGA2* involved fusion genes in PA, for example.

The most frequent cytogenetic aberration in MEC is the translocation t(11;19)(q21;p13). In 2003 Tonon and co-workers has first described the translocation associated fusion gene *CRTC1-MAML2*, in two MEC cell lines and three primary-tumor biopsy samples from MEC (Tonon et al., 2003). Behboudi and colleagues found the fusion transcript in 16 out of 29 fresh frozen MEC cases (55%). Of additional value from this study was the finding that the fusion protein was expressed in all MEC-specific cell types. The authors also reported cytogenetic data that suggested that not all fusion-positive tumors carried the t(11;19), with the implication that other cryptic translocations may contribute to the process in such cases (Behboudi et al., 2006). The *CRTC1-MAML2* transcript expression is not tissue-specific being detected in MEC arising in both minor and major salivary glands, lung, cervix and thyroid gland (Roser et al., 2007; Tirado et al., 2007; Lennerz et al., 2009). These findings suggest that in this tumor, *CRTC1-MAML2* acts as an early stage in tumor initiation. The alternative fusion gene *CRTC3-MAML2* provides further support for such a mechanism (Fehr et al., 2008b). A similar t(11;19)(q21;p13) translocation has also been identified in a subset of benign WATs (Bullerdiek et al., 1988; Mark et al., 1990). This translocation was shown to result in the same *CRTC1–MAML2* fusion in WAT like in MEC (Enlund et al., 2004; Tirado et al., 2007; Bell et al., 2008; Fehr et al., 2008a). WAT and MEC are both salivary gland

tumors but do not generally share clinicopathological features, and their accepted histogenesis is also distinct (Barnes et al., 2005; Simpson and Eveson 2005). Although the great majority of WAT are benign, with an excellent clinical outcome, malignant transformation is occasionally reported, including that to MEC (Williamson et al., 2000). However, metaplastic WAT is characterized by replacement of much of the original epithelium by metaplastic squamous cells, along with regressive changes in the stroma areas. Misinterpretation of metaplastic WAT for malignancy like squamous epithelium carcinoma (PEC) or MEC is a serious diagnostic pitfall (Di Palma et al., 1999; Schwerer et al., 2001).

CRTC1-MAML2 Fusion in WAT (Paper I)

To evaluate the incidence of the fusion *CRTC1-MAML2* in WAT, we have tested a series of 48 WAT by RT-PCR for the presence of the fusion gene. From our examined WAT offer 2 of 48 cases (4%) a *CRTC1-MAML2* fusion and this cases were the only metaplastic cases in the study, so the fusion seems to be a rare event in WAT and may be restricted to "non-typical" WAT (Fehr et al., 2008a). This observation is in accordance with Bell et al. (2008), who has analysed five cases of "non-typical" WAT, associated with malignancy (three cases of WAT with co-existent MEC; one WAT with co-existent metastatic melanoma; and one case of primary malignant WAT) for *CRTC1-MAML2* expression. All five cases studied were fusion positive. Specifically, all benign elements of WAT, the co-existent MEC and the malignant WAT were fusion positive whilst the melanoma was negative for the transcript. Of the three cases of WAT with co-existent MEC, metaplastic changes were noted in the oncocytic epithelium juxtaposed between the WAT and MEC (Bell et al., 2008). Bell and colleagues suggests that WAT and synchronous MEC share the same t(11;19) fusion gene and that this may constitute an early or initiating event in the clonal development of a subset of WATs. In addition to confirming the presence of the *CRTC1-MAML2* fusion gene in benign WATs, Bell and colleagues suggest that some of these lesions may be prone to malignant transformation. Together, these findings along with the simultaneous occurrence of both tumors and the sharing of identical cytogenetic and molecular findings support a histogenetic link between certain WATs and the development of MEC. Moreover, the detection of the fusion transcript in the Warthin's carcinoma extends the role of this genetic event to the direct malignant transformation of oncocytic epithelium (Bell et al., 2008). Our

data and the data from Bell and co-workers suggest that this transcript may be a factor in the pathogenesis of WAT, although at a lower prevalence than for MEC (Bell et al., 2008; Fehr et al., 2008a). This finding differs from previously published data from Tirado and colleagues, which found in 4 of 11 examined ordinary WAT the fusion gene, in addition in this study WAT with the fusion transcript were histologically identical to those lacking the *CRTC1-MAML2* transcript (Tirado et al., 2007). Enlund et al. has also detected the fusion in one ordinary WAT (Enlund et al., 2004).

Interestingly, an apparently identical translocation t(11;19)(q21;p13) has also been described in a third tumor type, in clear cell hidradenoma (CCH) of the skin (Gorunova et al., 1994). CCH is a benign tumor originating from intraepidermal sweat duct units. Sweat ducts are like salivary glands exocrine organs. RT-PCR analysis on a couple of CCHs revealed expression of a *CRTC1–MAML2* fusion transcript with the same fusion points as those found in both MEC and WAT (Behboudi et al., 2005; El-Naggar 2006; Winnes et al., 2007). These interesting observation demonstrate that the t(11;19)(q21;p13) in MEC, WAT and CCH target the same genes and that the *CRTC1–MAML2* fusion therefore cannot be considered to be tumor specific, like the *CTNNB1-PLAG1* fusion in PAs. Moreover, these studies reveal an important genetic link between MEC, WAT and CCH and suggest common molecular pathways that may be of importance for the development of both benign and malignant glandular tumors.

However, the impact of the *CRTC1-MAML2* fusion on the clinical outcome in WAT and CCH is unclear and controversial in the literature. Today we have not enough data about the *CRTC1-MAML2* fusion in CCH and in particular in WAT, so further studies are needed. Nevertheless, we suggest that all histological questionable cases of WAT and “non-typical” WAT exhibiting the *CRTC1-MAML2* fusion should be regarded with caution, and at least classified as indeterminate. So we recommend testing WAT for the presence of *CRTC1-MAML2* and monitoring patients with this fusion more closely; analogous to follow-up practices for other salivary gland adenomas at risk for recurrence or progression disease. Overall, our data clearly point to the t(11;19)(q21;p13) translocation with *CRTC1-MAML2* expression being a very rare event in WAT. It should be remembered that WAT is a relatively common salivary gland tumor and that malignant transformations remains rarely reported.

Further investigation of larger series of both ordinary WAT and those with any associated malignancy would be a great value.

CRTC1-MAML2 and *HMGA2* in MEC (Papers II, III and IV)

The main goals of our studies on MEC was it to establish a solid test system for FFPE material based on RT-PCR, to check the incidence of *CRTC1-MAML2* in MEC and to analyse if there are a correlation between the fusion status and the grade / prognosis of the tumor. The well known oncogene *HMGA2* is involved in the tumorgenesis of a couple of tumors, amongst others in PA and CaPA of the salivary gland (Geurts et al., 1997; Geurts et al., 1998; Persson et al., 2009). Hence, additionally to the *CRTC1-MAML2* status a couple of MECs should be analyzed for the expression of *HMGA2*, to investigate if there is a potential relationship between the *CRTC1-MAML2* fusion status, the tumor grade and the *HMGA2* expression level.

The limitations of conventional cytogenetic techniques that require the use of fresh tumor samples to determine the frequency of the t(11;19) in MEC have been overcome by the use of molecular techniques to detect the associated *CRTC1-MAML2 fusion* transcript. Using RT-PCR and / or fluorescence in situ hybridization (FISH) analyses. Within this thesis it was possible to establish a solid, RT-PCR-based, test system optimized for the detection of *CRTC1-MAML2* in FFPE tissue (Fehr et al., 2008b; Fehr et al., 2009). Our studies have shown a few low and intermediate grade tumors which are negative for the *CRTC1-MAML2* fusion (Fehr et al., 2008b; Fehr et al., 2009; Verdorfer et al., 2009); this is not remarkable, the main reason for this result could be the detection limit of the PCR reaction; Low-grade tumors are often characterized by a cystic structure and “non-tumor” cells without translocation, so in a few cases might be not enough *CRTC1-MAML2* positive cells for detection by RT-PCR, another reason could be the poor mRNA quality of the FFPE material. On the other hand we have also detected the fusion in a few high-grade tumors (Fehr et al., 2008b; Fehr et al., 2009; Verdorfer et al., 2009), here we have one principle problem of the histological classification of tumors, because there could be border cases which for one pathologist may be a high-grade tumor and for the next pathologist an intermediate-grade tumor. To determine the right grade is often a subjective decision, the same is true for in the diagnostic of salivary gland tumors specialized pathologists. These are exactly the cases were molecular

biomarkers like *CRTC1-MAML2* may helpful, to find the exact diagnosis (or tumor grade) and based on these an appropriate treatment. It is our contention that our test system for the detection of *CRTC1-MAML2* is a powerful tool for diagnoses and prognoses of MEC in FFPE and fresh material. Moreover, we suggest it may also practical for fine needle aspiration biopsy. A specific diagnosis by fine needle aspiration biopsy can only be made in approximately 60-75% of salivary gland cases (Ellis and Auclair 1996). False negative diagnoses due to inadequate sampling appear to be the most frequent errors, in this context our PCR basesd *CRTC1-MAML2* test may an appropriate tool for diagnoses, because the test needs only a few tumor cells to work. The PCR assay is used since a few years successfully in the routine analysis of MEC in the Institute of Haematopathology and the Albertinen-Pathology (both Hamburg). With this technique, we were able to screen a series of approximately 140 MEC tumors for evidence of a *CRTC1-MAML2* fusion.

In our MEC studies (Fehr et al., 2008b; Fehr et al., 2009; Verdorfer et al., 2009), about 70% of the tested FFPE tumors showed evidence of a *CRTC1-MAML2* fusion, in agreement to previous reports (Enlund et al., 2004; Martins et al., 2004; Tirado et al., 2007). This is a very high detection rate for the fusion in FFPE material. Recent studies indicate the presence of *CRTC1-MAML2* in 55–81% of MEC for fresh-frozen samples (Martins et al., 2004; Behboudi et al., 2006; Tirado et al., 2007) and approximately 35% for formalin-fixed samples (Okabe et al., 2006; Miyabe et al., 2009). In the latter study the authors commented that the differences in the detection rates across these studies may reflect the nature of the tumor material studied, with lower rates of detection being reported when archival paraffin-embedded tumor was analysed in comparison to studies using fresh-frozen tissue (Tirado et al., 2007).

Nevertheless, we have shown that our test system has the same, or nearly the same detection efficient for FFPE material, as described by other authors for fresh-frozen material (Enlund et al., 2004; Martins et al., 2004; Tirado et al., 2007; Bell et al., 2008). Additionally, in one case we have found *CRTC3* as an alternative fusion partner of *MAML2* (Fehr et al., 2008b). CRTC3 is the third known fusion partner of MAML2. In previous studies the same part of MAML2 has been reported to be fused in CRTC1-MAML2 (Tonon et al., 2003) as well as to the N-terminal part of MLL in secondary acute myeloid leukemia and myelodysplastic syndrome (Nemoto et al., 2007). Since CRTC1 shares 32% amino acid identity with CRTC2 and CRTC3, we suggest similar functions for the three proteins. This new fusion seems to be also

associated with a favorable prognosis for the patient like those for *CRTC1-MAML2* positive cases. However the patients may be younger than those with *CRTC1-MAML2* fusion or those with no detectable gene fusion (Nakayama et al., 2009). Hence, it is reasonable to expect that CRTC3-MAML2 fusion genes will have similar input on the tumor phenotype.

Furthermore in our studies the fusion gene was mainly found in low- and intermediate-grade tumors (Fehr et al., 2008b; Fehr et al., 2009; Verdorfer et al., 2009), as already recommended by others (Behboudi et al., 2006; Okabe et al., 2006). On the other side *CRTC1-MAML2* negative tumors were mainly found in high-grade tumors and show a significant increased *HMGA2* level. Our statistical analyses showed a significant discrepancy in the *HMGA2* expression level between *CRTC1-MAML2* positive and negative tumors (Fehr et al., 2009). In addition, there is a statistical significant variation between the *HMGA2* medians of the three grading stages. Behboudi et al. showed that the clinical prognosis for low-grade MECs of the salivary glands was much better than for high-grade tumors (Behboudi et al., 2006), and Tirado et al., described *CRTC1-MAML2* negative tumors to be more likely to develop the distant metastases (Tirado et al., 2007) that characterize a highly aggressive tumor. Several authors have described a strong association between the overexpression of *HMGA2*, the malignant phenotype and an adverse prognosis of carcinomas (Rogalla et al., 1997; Rommel et al., 1997; Rogalla et al., 1998; Sezer et al., 2000; Gross et al., 2003; Langelotz et al., 2003; Miyazawa et al., 2004; Sarhadi et al., 2006; Meyer et al., 2007a; Meyer et al., 2007b; Winkler et al., 2007; Belge et al., 2008), so our investigation about the increased *HMGA2* level correlate well with the adverse prognosis for high-grade MEC. The histopathologic grade of a MEC is an established predictor of prognosis and treatment. Auclair and Goode found that the 5-year mortality rates for MECs were 3%, 10% and 46% for low-grade, intermediate-grade and high-grade tumors, respectively (Auclair et al., 1992; Goode et al., 1998). There is a high correlation between the grading and the long-term survival rate. Because of the more aggressive behavior of high-grade compared to low-grade MECs, a more intensive treatment has been recommended for the former group (Nance et al., 2008). Our studies support this statement because the results showed that the level of *HMGA2* expression correlated with the tumor grade.

These data raise doubts about the correct classification of MECs. The present classification of MEC includes two subgroups: a huge group of “true-MECs” with the

CRTC1-MAML2 fusion (with a moderate aggressiveness and an excellent prognosis), and a smaller group of morphology heterogeonus tumors lacking this fusion (more aggressive and with a worse prognosis). We conclude that the second group, which lacks the fusion, may represent a distinct category of poorly differentiated carcinomas of non-MEC etiology with aberrantly high *HMGA2* levels. The proportion of different cells types (squamous, mucous and intermediate) and their architectural configuration varies within and between MECs. For the differential diagnosis these variances of the MEC architecture are very problematical because the single cell types could also occur in other tumor types. This complicates a correct diagnosis and the prognosis of the tumor (Ellis and Auclair 1996; Goode and El Naggar 2005; Luna 2006). We suggest that this non-MEC group envelops not only one specific tumor type in fact it seems to be a mix of different tumor types, all poorly differentiated with typical high-grade features (squamous and / or solid structure, absence of mucous-producing cells) and a high HMGA2 expression level. Therefore, these group of non-MEC tumors may cover tumors which are also included in the differential diagnosis of MEC, like adenosquamous carcinoma, or squamous cell carcinoma for example (Goode and El Naggar 2005). Another tumortype in this group could be CaPA, with a poorly differentiated carcinoma part. Persson et al. have hypothesized that amplification of *HMGA2* may cause the malign transformation of the benign PA to a CaPA (Persson et al., 2009). Maybe there is a similar mechanism of transformation in these non-MEC tumors.

These two groups might have different origins, with the first true-MEC group characterized by a *CRTC1-MAML2* fusion may representing a histogenetic link between Warthin's tumors and the development of MEC as supposed by Bell (Bell et al., 2008). One possible origin for the non-MEC group could be the translocation t(3;8)(p21;q12) described by Bullerdiek et al. in a MEC (Bullerdiek et al., 1990). Interestingly this translocation and an aberration of the region 12q14 with the *HMGA2* gene are often described in the context of PAs of the salivary gland (Mark and Dahlenfors 1986; Mark et al., 1988; Fonseca et al., 2008; Persson et al., 2009).

In our opinion, the non-MEC group is of special importance for future investigations, providing prognostic and particularly therapeutic relevance. We presume based on our study that the elevated mRNA levels are as a rule due to a global dedifferentiation observed in the tumors rather than to mutations affecting HMGA2 (Fehr et al., 2009). This was suggested by the result of FISH analyses on a few of

the tumors, with and without the *CRTC1-MAML2* fusion, using a HMGA2 break-apart probe, which we have performed (data not shown). The FISH analysis showed neither amplification nor rearrangements of the HMGA2 locus. However, we cannot rule out minor changes of the HMGA2 locus which the FISH break-apart probe cannot detect. Nevertheless, the general association between high *HMGA2* level and the aggressiveness of solid tumors could be an interesting starting point for a possible *HMGA2* silencing therapy; previous reports have discussed this form of therapy for thyroid neoplasms, liposarcomas and ovarian cancer. The authors have silenced the *HMGA2* gene, both *in vitro* and *in vivo*, which results in growth inhibition of the tumor and increased apoptosis (Berlingieri et al., 1995; Pentimalli et al., 2003; Malek et al., 2008).

Finally, on the basis of our studies on MEC we approve that all MEC tumors should be analyzed for the *CRTC1-MAML2* fusion in the future routinely. In particular in border cases (intermediate vs. high-grade) a *CRTC1-MAML2* test may be very useful to do a correct diagnosis and an adapted treatment.

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Appendix

Abbreviations

AdCC	Adenoid cystic carcinoma
aCGH	Microarray-based comparative genomic hybridization
AFIP	Armed Forces Institute of Pathology
CaPA	Carcinoma ex pleomorphic adenoma
CCH	Clear cell hidradenoma of the skin
CHCHD7	Coiled-coil-helix-coiled-coil-helix domain containing protein 7
CTNNB1	β-catenin gene
DNase	Deoxyribonuclease
EST	Expressed sequence tag
FFPE	Formalin fixed and paraffin embedded
FHIT	Fragile histidine triad gene
FISH	Fluorescence in situ hybridization
GAPDH	Glyceraldehyde-3-hosphate dehydrogenase
HMGA2	High-mobility group AT-hook 2
LIFR	Leukemia inhibitory factor receptor
MEC	Mucoepidermoid carcinoma
NCBI	National Center for Biotechnology Information
NFIB	Nuclear factor 1/B
ORF	Open reading frame
PA	Pleomorphic adnoma
PCR	Polymerase chain reaction
PLAG1	Pleomorphic adenoma gene 1
RNase	Ribonuclease
RT	Room temperature
SDS	Sodium dodecyl sulfate
TCEA1	Transcription elongation factors A1
U	Unit
UKE	Universitätsklinikum Hamburg-Eppendorf
UNG	Uracil-N-glycosylase
WAT	Warthin's tumor (cystadenolymphoma)
WHO	World Health Organisation

The international system (SI) of units is valid for all other abbreviations.

Distributors

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Erklärung

„Hiermit erkläre ich, André Fehr, geboren am 19. November 1975 in Bremen, dass ich die vorliegende Dissertation „Untersuchungen zur Genetik von Speicheldrüsentumoren“ selbstständig verfasst und keine anderen, als die angegebenen Quellen und Hilfsmittel verwendet habe“

Göteborg, den 27.09.2009 _____

(André Fehr)

Papers I – IV

I. **Fehr A*, Röser K*, Belge G, Löning T, Bullerdiek J.** 2008a. A closer look at Warthin tumors and the t(11;19). Cancer Genet Cytogenet 180(2):135-9.

II. **Fehr A, Röser K, Heidorn K, Hallas C, Löning T, Bullerdiek J.** 2008b. A new type of *MAML2* fusion in mucoepidermoid carcinoma. Genes Chromosomes Cancer 47(3):203-6.

III. **Verdorfer I, Fehr A, Bullerdiek J, Scholz N, Brunner A, Krugmann J, Hager M, Haufe H, Mikuz G, Scholtz A.** 2009. Chromosomal imbalances, 11q21 rearrangement and *MECT1-MAML2* fusion transcript in mucoepidermoid carcinomas of the salivary gland. Oncol Rep 22(2):305-11.

IV. **Fehr A, Meyer A, Heidorn K, Röser K, Löning T, Bullerdiek J.** 2009. A link between the expression of the stem cell marker *HMGA2*, grading, and the fusion *CRTC1-MAML2* in mucoepidermoid carcinoma. Genes Chromosomes Cancer 48(9):777-85.

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I

Fehr A*, Röser K*, Belge G, Löning T, Bullerdiek J. 2008a. A closer look at Warthin tumors and the t(11;19). Cancer Genet Cytogenet 180(2):135-9.

Contribution to the work

- Planning of all done work with Dr. Kerstin Röser
- Performance of all done work
- Composing of the publication assisted by Dr. Kerstin Röser

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A closer look at Warthin tumors and the t(11;19)

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Abstract The translocation t(11;19)(q21;p13) has been described in mucoepidermoid carcinoma (MEC) and rarely in Warthin tumors (WT), both tumors of the salivary gland. The translocation creates a fusion gene in which exon 1 of *CRTC1* is linked to exons 2–5 of *MAML2*. To verify the translocation in WT, we performed nested reverse transcriptase–polymerase chain reaction using RNA from 48 WTs. This revealed the t(11;19)(q21;p13) translocation and expression of the chimeric gene in two metaplastic WT samples, but in none of the remaining ordinary 46 WTs. On review, the two positive cases were classified as tumors highly suspect for MEC. Indeed, our experience and published observations of the t(11;19)(q21;p13) translocation in WT reveal that only a small subset of WTs are positive, and that these tumors are often classified as infarcted or metaplastic WT, known to overlap considerably with MEC on purely morphological grounds. We therefore conclude that the presence of the t(11;19)(q21;p13) rearrangement favors a diagnosis of MEC.

1. Introduction

Mucoepidermoid carcinoma (MEC) is the most frequent malignant tumor type of the salivary glands and the second most frequent lung tumor of bronchial gland origin [1,2]. Previous cytogenetic studies on MECs of the salivary and bronchial glands have revealed a t(11;19)(q21;p13) translocation, in some cases as the only chromosomal rearrangement [3–5]. The translocation was first described by Bullerdiek et al. [6] in a Warthin tumor.

The second most common benign tumor of the salivary glands [7], WT is composed of glandular and often cystic structures, sometimes with a papillary cystic arrangement, lined by characteristic bilayered epithelium comprising inner columnar eosinophilic or oncocytic cells surrounded by smaller basal cells. The stroma contains a variable amount of lymphoid tissue with germinal centers. Most WTs are well circumscribed and partly cystic. The cysts vary from small slits to quite large spaces and contain clear, mucous, creamy white or brown fluid. In the metaplastic variant, solid areas are predominant and often firm and fibrous. The immunoprofile of the lymphocyte subsets is similar to that in normal or reactive lymph nodes [8,9]. The typical type of WT is usually unmistakable in differential diagnostics. In cases of marked regressive changes and subsequent squamous and mucous metaplasia (the so-called metaplastic variants), however, the differential diagnosis of MEC invariably occurs [10,11].

Recent studies have shown that the t(11;19)(q21;p13) translocation in MEC and sometimes in WT results in a fusion of the N-terminal domain of the CREB-regulated transcription coactivator 1, *CRTC1* (previously *MECT1*; alias *TORC1*, *WAMTP1*), with the Notch coactivator *MAML2* (mastermind-like 2) [12–14]. Exon 1 from *CRTC1* at 19p13 fuses with exons 2–5 of *MAML2* at 11q21. Full-length *MAML2* acts as a CSL-dependent transcriptional coactivator of Notch. In contrast, *CRTC1–MAML2* activates transcription of the Notch target gene *HES1* on 3q28~q29, independent of Notch ligands and CSL binding sites. In addition, the recent identification of the *CRTC1* gene product as a potent coactivator for genes that are regulated by cAMP-responsive elements suggests that *CRTC1–MAML2* may be disrupting both Notch and CREB signaling pathways to induce tumorigenesis [15–17].

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To check for the t(11;19)(q21;p13) translocation in WT that could result in a *CRTC1–MAML2* gene fusion, we performed reverse transcriptase–polymerase chain reaction (RT-PCR) using RNA from 48 WTs.

2. Materials and methods

In this study, 46 snap-frozen WTs, two formalin-fixed, paraffin-embedded (FFPE) metaplastic WTs, and one human MEC cell line (NCI-H292) were used. All tumors were obtained from the Salivary Gland Registry in Hamburg, Germany. All tumors were carefully reexamined, and the diagnosis of WT was confirmed according to the criteria of the World Health Organization [9].

Total RNA from snap-frozen tumors and NCI-H292 was extracted by using TRIzol LS reagent (Invitrogen, Karlsruhe, Germany). For cDNA synthesis, 5 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany), using a poly(A)-oligo(dt)17 primer. For RNA extraction of the two FFPE WTs, we used six 5-μm sections of FFPE tissue. The tissue was deparaffinated and the total RNA was isolated by using a High Pure RNA paraffin kit according to the manufacturer's instructions (Roche Diagnostics, Penzberg, Germany).

For cDNA synthesis, 1 μg of total RNA was used for reverse transcription using M-MLV reverse transcriptase with random primers according to the manufacturer's instructions (both Invitrogen, Karlsruhe, Germany). As a control for RNA quality, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was evaluated on all cDNAs used, as previously described [18]. Amplification with these primers yielded a 445-bp PCR product.

All snap-frozen WTs were screened for expression of *CRTC1–MAML2* fusion transcript by nested RT-PCR. The first PCR amplification of the tumor cDNA was performed in a final volume of 50 μL containing 3 μL template cDNA, 5 μL 10× PCR buffer without Mg^{2+}, 0.5 μmol/L of each primer, 200 μmol/L dNTP mix, 1.5 mmol/L $MgCl_2$, and 0.5 μL *Taq* DNA polymerase (5 U/μL) (Invitrogen, Karlsruhe, Germany). The following primer sequences were used for the first PCR: (forward 1) 5′-GACTTCGAACAATCCGCGGAAAT-3′ and (reverse 1) 5′-TGAAGGGATTGGAGACGAAGTGGA-3′. The oligonucleotide primers were designed to amplify a segment, ranging from exon 1 of the *CRTC1* gene to exon 2 of the *MAML2* gene, with an expected size of 826 bp.

Two microliters product (diluted 1:10) of each probe from the first PCR reaction was used as template for a second amplification using nested primer (forward 2) 5′-GCCTTCGAGGAGGTCATGA-3′ and (reverse 2) 5′-CTTGCTGTTGGCAGGAGA-3′. These oligonucleotide primers amplify a segment with an expected size of 105 bp. Both PCR reactions were performed using a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). First-round and nested RT-PCR were performed after an initial denaturation at 94°C for 3 minutes over 35 cycles of amplification, using the following conditions: 94°C for 45 seconds, 62°C for 30 seconds, and 72°C for 1 minute. For nested PCR, an annealing step for 30 seconds at 55°C and an extension step for 30 seconds at 72°C were performed. PCRs were completed with a final extension for 10 minutes at 72°C.

The RT-PCR amplification of cDNA from the two FFPE cases was performed as for the second PCR for the snap-frozen tumors, but with 10 μL template cDNA.

In addition, we repeated the PCR with 5 μL template cDNA and performed a Southern blot with a *CRTC1–MAML2* specific probe. The PCR product was separated by electrophoresis in a 2% agarose gel and transferred to nylon membrane Hybond N+ (Amersham, Buckinghamshire, UK). The blot was hybridized with a probe from the *CRTC1–MAML2*-positive cell line NCI-H292, labeled by digoxigenin-11-dUTPs (Roche Diagnostics, Penzberg, Germany). The labeling reaction was performed similarly to the first PCR. For prehybridization and hybridization, the ExpressHyb hybridization solution (Clontech Laboratories, Mountain View, CA) was used. Before hybridization, 250 ng of the probe was denatured at 95°C for 5 minutes. Prehybridization was performed for 1 hour and hybridization for 16 hours at 62°C. Blot were washed twice for 10 minutes at room temperature in 2× saline sodium citrate (SSC)–0.1% sodium dodecyl sulfate (SDS) and twice for 15 minutes at 62°C in 0.5× SSC–0.1% SDS. Further steps with blocking reagents, anti-digoxigenin-AP and CDP-Star (all Roche Diagnostics, Penzberg, Germany), were performed according to the manufacturer's instructions.

3. Results

As control for intact RNA and cDNA, a RT-PCR for the housekeeping gene *GAPDH* was performed on all cDNAs used. The 445-bp product for *GAPDH* was detected in all samples (Fig. 1; not all data shown). To investigate the expression of the chimeric gene in WT specimens, we used RT-PCR to screen a total of 48 WTs and one cell line for presence of the *CRTC1–MAML2* fusion gene. A fusion transcript was detected in the NCI-H292 cell line and in both metaplastic WTs, but in none of the 46 ordinary WTs (Figs. 2a and 2b; not all data shown). The Southern blotting results confirmed the two positive metaplastic WTs (Figs. 2c and 2d).

4. Discussion

WT and MEC are both salivary gland tumors but do not generally share clinicopathological features, and their accepted histogenesis is also distinct [9,19]. Metaplastic WT, however, is characterized by replacement of much of

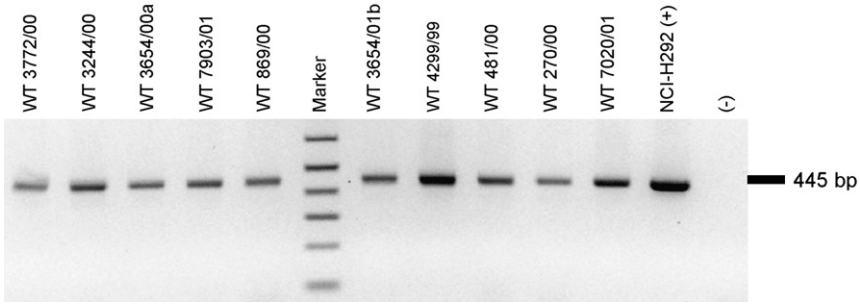

Fig. 1. Reverse transcriptase–polymerase chain reaction (RT-PCR) products of *GAPDH* as control for intact RNA and cDNA. The 445-bp product for *GAPDH* was detected in all Warthin tumor samples (not all data shown).

the original epithelium by metaplastic squamous cells, along with regressive changes in the stroma areas (Fig. 3). Misinterpretation of metaplastic WT as a malignancy such as squamous epithelium carcinoma (PEC) or MEC presents a serious diagnostic pitfall [10,11]. The translocation t(11;19)(q21;p13) and the *CRTC1–MAML2* chimeric gene have been described in MEC [12], in some WTs [7], and in some clear cell hidradenomas (CCH) [20,21]. To date, however, there was only one previously reported WT with a *CRTC1–MAML2* expression and a t(11;19)(q21;p13) translocation respectively [13,22], and one WT with a complex translocation in t(11;16;19)(q21;p13.3;p12~13) that contains the *CRTC1–MAML2* chimeric gene, but this case was reclassified as a MEC ex-WT [14].

To evaluate the incidence of t(11;19)(q21;p13) translocation in WT, we used RT-PCR to test a series of 48 WTs for the presence of the *CRTC1–MAML2* chimeric gene. Also included was the MEC cell line NCI-H292, which expresses chimeric gene. In none of the 46 ordinary tumors was the *CRTC1–MAML2* chimeric gene expressed. In the typical WT, the diagnosis is usually straightforward, as it was for the series of ordinary tumors. In the setting of the metaplastic variants, however, MEC can hardly be sorted out purely on morphological grounds. In fact, the primary diagnosis of our two cases was metaplastic WT, yet on review the tumors were reclassified as highly suspect for MEC. The *CRTC1–MAML2* fusion transcript could be detected in both lesions. To further validate this result, we

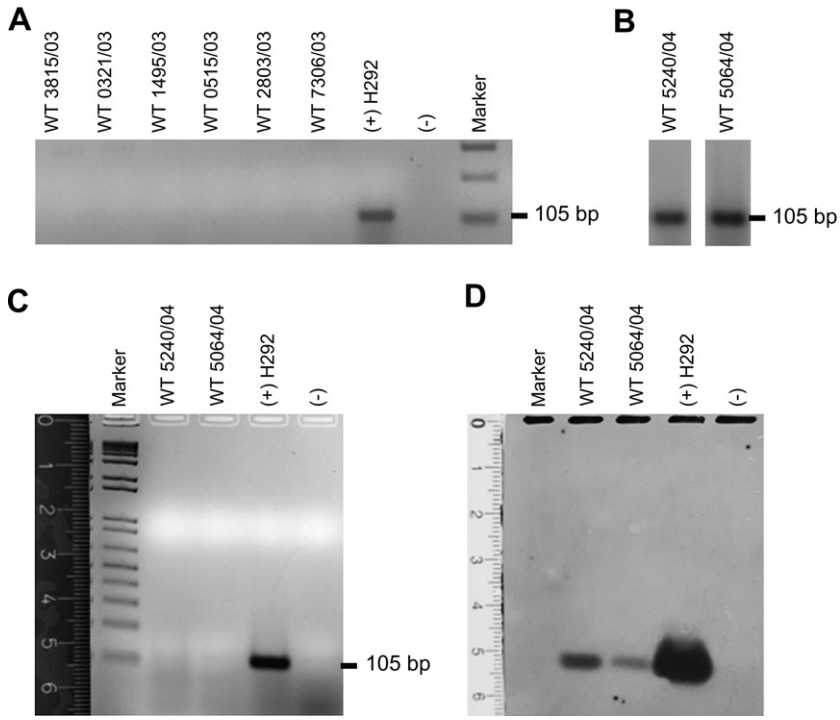

Fig. 2. RT-PCR (A,B) and Southern blot (C,D) experiments for detection of the *CRTC1–MAML2* chimeric transcript. (A) PCR results for selected Warthin tumor (WT) samples, out of 46 WTs all negative for the chimeric transcript, and cell ine NCI-H292, the positive control. Not all data are shown. (B) PCR results for two metaplastic WTs, both of which contain the chimeric transcript. (C) Blotting gel with the two metaplastic WTs and (D) blotting membrane after hybridization and detection with a probe specific for *CRTC1–MAML2*. Both metaplastic WTs are positive for the fusion gene.

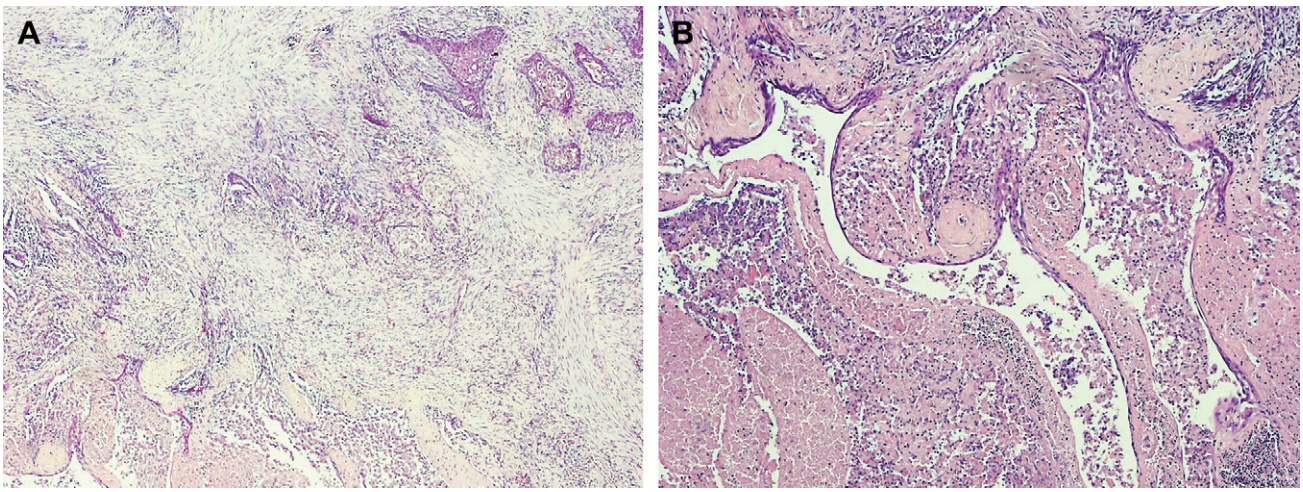

Fig. 3. Metaplastic Warthin tumor (WT) tissue sample, case WT 5064. (A) Typical (low power) view of metaplastic WT with squamous islands in the upper right part of the figure; note extensive necrosis at the bottom (hematoxylin–eosin-stain; ×100). (B) Higher magnification view of the cystic and necrotic area of the tumor reveals covering epithelia with abortive squamous metaplasia, but without straightforward mucinous differentiations (hematoxylin–eosin-stain; ×250).

repeated the PCR and performed a Southern blot with a probe specific for *CRTC1–MAML2*.

Our finding that all 46 tumors with a clear-cut diagnosis of WT were negative for the t(11;19)(q21;p13) translocation supports the concept that at least ordinary MEC and WT are genetically distinct. We suggest that all histologically questionable cases of WT and metaplastic WT exhibiting the *CRTC1–MAML2* chimeric gene should be regarded with caution, and at least classified as indeterminate. Overall, our data clearly point to the t(11;19)(q21;p13) translocation with *CRTC1–MAML2* expression being a very rare event in metaplastic WT and ordinary WT [13,22]. When the fusion gene is present in this tumor type, it seems to be restricted to special cases with indeterminate morphology, especially involving necrosis and subsequent metaplasia.

We recommend testing these tumors for the presence of the t(11;19)(q21;p13) translocation by RT-PCR and monitoring patients with these tumors more closely, analogous to follow-up practices for other salivary gland adenomas at risk for recurrence or progression (examples include pleomorphic adenomas with extensive necrosis and atypical adenomas, among others). Molecular markers such as the *CRTC1–MAML2* fusion gene can help in accurately classifying the tumors. In turn, precision in diagnosis makes it possible to refine the adjustment of therapy.

Acknowledgments

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Contribution to the work

- Planning and performance of all done work, except the sequencing reaction (Sequencing was performed by Dr. Cora Hallas and Dr. Klaus Heidorn)
- Composing of the publication

A New Type of *MAML2* Fusion in Mucoepidermoid Carcinoma

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The present study reports for the first time a *CRTC3-MAML2* fusion gene in a mucoepidermoid carcinoma, as determined by RT-PCR and sequencing. We screened a total of 67 formalin-fixed, paraffin-embedded mucoepidermoid carcinomas for the presence of chimeric genes. In one of these samples, a *CRTC3-MAML2* fusion gene was detected. Thus, this report demonstrates the existence of a fusion of *MAML2* with *CREB regulated transcriptional coactivator CRTC3* additional to the already known fusion of *MAML2* and *CRTC1*. Both gene fusions seem to result in an identical tumor phenotype and the fusion genes *CRTC1-MAML2* and *CRTC3-MAML2* may play a similar role in the development of mucoepidermoid carcinomas.

INTRODUCTION

Since the first discovery of the translocation t(11;19)(q21;p13) in a Warthin's tumor (Bullerdiek et al., 1988), it has been found to be a frequent cytogenetic aberration in mucoepidermoid carcinomas (MEC) with a frequency of about 40–50% (Behboudi et al., 2006; Okabe et al., 2006). Recent cloning of the translocation in MEC cell lines identified a fusion gene comprised exon 1 of the *CREB regulated transcriptional coactivator CRTC1* (also known as *MECT1*, *TORC1*, or *WAMTP1*) gene on chromosomal band 19p13 and exon 2–5 of the Mastermind-like gene *MAML2* on chromosomal band 11q21 (Tonon et al., 2003; Enlund et al., 2004).

Previous functional studies have shown that the expression of *CRTC1-MAML2* is essential for the growth of t(11;19) positive MEC cell lines and that the N-terminal CREB binding domain is fundamental for the transforming activity of the chimeric protein (Coxon et al., 2005; Wu et al., 2005; Komiya et al., 2006). Similar to *Drosophila Mastermind* and *MAML1*, full-length *MAML2* acts as a CSL-dependent transcriptional coactivator of Notch (Wu et al., 2000, 2002; Lin et al., 2002). In contrast, CRTC1-MAML2 activates the transcription of Notch target genes independent of Notch ligands and CSL binding sites. In addition, the recent identification of the CRTC1 protein as a potent coactivator for genes that are regulated by cAMP responsive elements suggests that *CRTC1-MAML2* may disrupt both Notch and CREB signaling pathways and induce tumorigenesis (Conkright et al., 2003; Iourgenko et al., 2003; Wu et al., 2005).

Here we show for the first time that *MAML2* not only fuses with *CRTC1* but also with *CRTC3* in MECs.

MATERIAL AND METHODS

Tumor Samples

Formalin-fixed, paraffin-embedded (FFPE) tissues from MECs were obtained from the Albertinen-Pathology (Hamburg, Germany) and the Salivary Gland Registry of Oral Pathology at the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). The grading of the tumors was 52% low-grade (G1), 32% medium-grade (G2), and 16% high-grade (G3). The mean age of the patients in this study was 52 years, and no gender bias was seen. All tumors were carefully reexamined and the diagnosis of MEC confirmed according to the criteria of the World Health Organization (Goode and El Naggar, 2005; Luna, 2005).

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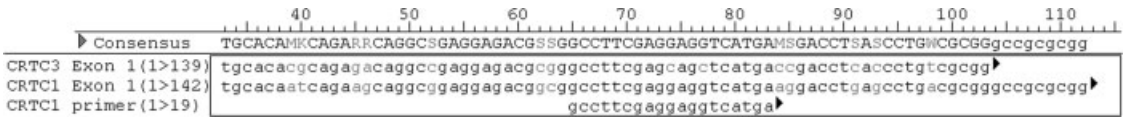

Figure 1. Alignment of primer *CRTC1* with exon 1 sequence from *CRTC1* and *CRTC3*. The primer for *CRTC1* binds with two mismatches (gray) to *CRTC3* sequence, enabling the amplification of a 98 bp fragment in combination with the primer *MAML2*.

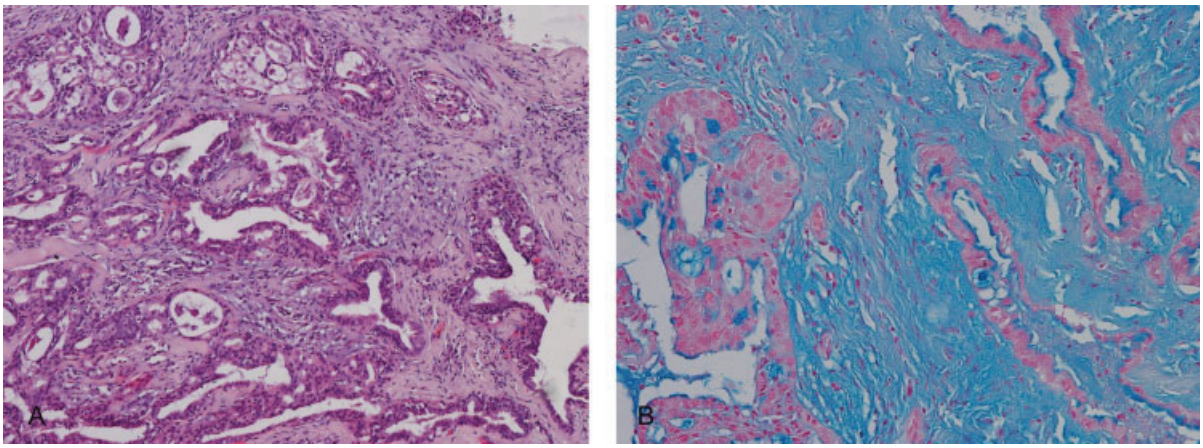

Figure 2. Sample 167, a highly differentiated tumor with cystic architecture and dispersed mucus containing cells (a) Haematoxylin-Eosin (×250) and (b) Alcian-Blue (×400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Transcript Analysis

Total RNA was extracted from FFPE tissue using the RNeasy FFPE Kit (Qiagen, Hilden, Germany). After elution of RNA with 30 µl of RNase-free water, 1 µl of total RNA was reverse transcribed and amplified by PCR using the one step RT-PCR Kit according to the manual (Qiagen, Hilden, Germany). Primers used for the *CRTC1-MAML2* PCR were *CRTC1* 5′-GCCTTCGAGGAGGTCATGA-3′ and *MAML2* 5′-CTTGCTGTTGGCAGGAGA-3′. The primers were designed to amplify a fragment extending from exon 1 of the *CRTC1* gene to exon 2 of the *MAML2* gene, with an expected size of 105 bp. The primer *CRTC1* is not only specific for *CRTC1*, but also binds to *CRTC3* cDNA with just two mismatches, enabling the amplification of a 98 bp fragment in combination with the primer *MAML2* (Fig. 1).

For specific detection of *CRTC3-MAML2*, we generated a *CRTC3* primer 5′-CGCGGAAGTTCAGTGAGA-3′, which together with the *MAML2*-primer, amplifies a product reaching from exon 1 of *CRTC3* to exon 2 of the *MAML2* gene, with an expected size of 155 bp. To check the quality of the cDNA we also amplified the wild type *MAML2* gene in every sample, using it as an internal control. To this purpose, we used the primers forward 5′-GTAGCAATAATGGTGGCAGT-3′ and reverse 5′-CTTGCTGTTGGCAGGAGA-3′. PCR reactions were run on an ABI2720 Thermal Cycler (Applied Biosystems, Foster City, USA) using the following conditions: after an initial denaturation step at 94°C for 3 min 35 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 30 sec were run followed by a final extension step of 10 min at 72°C. The PCR products were separated and sequenced using the ABI BigDye Terminator v1.1 sequencing kit (Applied Biosystems) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The nucleotide BLAST database (www.ncbi.nlm.nih.gov/BLAST/) was used for comparison of the sequencing data.

RESULTS

From a total of 66 MECs, 71% showed evidence of a *CRTC1-MAML2* fusion. The fusion gene was mainly found in low-grade (59%) and medium-grade (30%) tumors; only 11% from the *CRTC1-MAML2* positive samples were high-grade tumors. Additionally, a highly differentiated MEC (sample 167, Fig. 2) from a 61-year-old woman revealed an aberrant amplification product. Sequencing analysis demonstrated a fusion of *MAML2* with the *CRTC3* gene instead of *CRTC1*. To further validate this result, the RT-PCR was repeated using a primer specific for *CRTC3*.

Figure 3. (a) The nucleotide sequence of the *CRTC3-MAML2* breakpoint (GenBank accession number EU_048224); the primers are underlined (/ = breakpoint). (b) BLASTn results of sample 167 sequence: *CRTC3* part of sequence and (c) *MAML2* part of sequence. Both sequence-parts are 100% homolog to *CRTC3* and *MAML2*, respectively (http://www.ncbi.nlm.nih.gov/BLAST/, June 2007).

The sequencing analysis of the fusion product of the PCR with primers for *CRTC3* and *MAML2* revealed 97 nucleotides from exon 1 of *CRTC3* (GenBank accession number NM_022769.2) merged to 58 nucleotides from exon 2 of *MAML2* (GenBank accession number NM_032427.1). The nucleotide sequence of the breakpoint is given in Figure 3a.

A comparison of the fusion sequence obtained with the NCBI Nucleotide BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/, June 2007) is summarized in Figures 3b and 3c. The reading frame of the two genes is not affected by the translocation and a functional protein may be obtained from the sequence.

DISCUSSION

The present study shows that CRTC3 is an alternative fusion partner of MAML2 in MECs. Until now, a *CRTC3-MAML2* fusion gene, indicating most likely a translocation t(11;15)(q21;q26), has not been described in MECs or any other tumor entity (Mitelman Database, 2007). However, four salivary gland tumors and 268 other tumors are listed with an involvement of 15q26.

CRTC3 is the third known fusion partner of MAML2. The same part of MAML2 has been reported to be fused in CRTC1-MAML2 (Tonon et al., 2003) as well as to the N-terminal part of MLL in secondary acute myeloid leukemia and myelodysplastic syndrome (Nemoto et al., 2007).

We screened a series of 67 tumors for evidence of *CRTC1-MAML2* and *CRTC3-MAML2*, but found the aberration *CRTC3-MAML2* fusion in only one of the samples. Among the remaining 66 MECs, 71% showed evidence of a *CRTC1-MAML2* fusion, in agreement to previous reports from Enlund and Martins et al. (Enlund et al., 2004; Martins et al., 2004). The fusion gene was mainly found in low grade tumors as already suggested by others (Behboudi et al., 2006; Okabe et al., 2006).

CRTC1 has been identified as a candidate gene for the induction of salivary gland tumors (Tonon et al., 2003). The t(11;19), seen in MECs creates a fusion protein comprising the N-terminal CREB binding region of CRTC1 and the transcriptional activation domain of MAML2. The resulting fusion protein, CRTC1-MAML2, binds to CREB, activates cAMP-response element mediated transcription and induces formation of transformed RK3E cell foci (Conkright et al., 2003; Wu et al., 2005).

The CRTC1-MAML2 fusion protein lacks, however, the SIK (salt-inducible kinase) phosphorylation site of CRTC1, a site highly conserved in the whole *CRTC* family (Katoh et al., 2006). Phosphorylation of this site leads in the wild type gene to repression of CRTC1 activity as a transcriptional activator. Loss of this site in the fusion gene may induce permanent activity of the fusion protein and may thereby cause the uncontrolled transcription of CRE dependent elements, possibly contributing to tumor formation in salivary glands.

Since CRTC1 shares 32% amino acid identity with CRTC2 (chromosome 1q21.3) and CRTC3 (chromosome 15q26.1), similar functions are suggested for the three proteins (Iourgenko et al., 2003). Hence, it is reasonable to expect that

CRTC3-MAML*2* fusion genes will have similar input on the tumor phenotype.

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III

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Contribution to the work

- Coordination of the cooperation
- Performance of the *CRTC1-MAML2* RT-PCR
- Assistance at the composing of the publication

Chromosomal imbalances, 11q21 rearrangement and MECT1-MAML2 fusion transcript in mucoepidermoid carcinomas of the salivary gland

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Abstract. The aim of this study was to determine genetic alterations in mucoepidermoid carcinomas of the salivary gland in association with clinical and histopathological parameters. Nineteen formalin-fixed, paraffin-embedded tumors were analysed by using comparative genomic hybridization (CGH), fluorescence *in situ* hybridization (FISH) on interphase nuclei and reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of *MECT1-MAML2* fusion transcript. The CGH analysis showed an overrepresentation of chromosome X and losses of entire chromosomes or regions on chromosome 1, 2, and 15 as the most frequent copy number changes. In 37% of the analysed tumors a *MAML2*-rearrangement by interphase FISH was detected, whereas 58% of the samples showed expression of *MECT1-MAML2* fusion transcript. We conclude that the presence of MAML2-rearrangement as well as of *MECT1-MAML2* fusion transcript may reflect a more favourable prognosis and may be a useful marker for clinical prediction of the biological behavior of these tumors as previously reported.

Introduction

Mucoepidermoid carcinomas (MECs) are rare malignant neoplasms of variable histopathologic differentiation with unpredictable clinical behavior. These tumors showed a wide age distribution with an incidence peak at about the fifth decade of life. MECs are composed of three different cells types: intermediate, epidermoid (squamous) and mucus-secreting cells (1). Approximately half of the tumors occur in the parotid glands. Patients with a high grade carcinoma have an unfavorable outcome; however, the clinical and prognostic impacts of molecular aberrations remain unknown, due to the limited number of reported cases in the literature. Genetic analyses on MECs like G-banding, FISH, SKY and CGH revealed genetic losses at chromosome 9p21, 8p, 5p, 16q and 12p and gains of 7 (2-7).

A specific translocation t(11;19)(q21;p13) is known, which is associated with two types of salivary gland tumors, namely MECs as well as Warthin's tumors (8,9). The Warthin's tumor, a likewise frequently more occurring benign tumor of the salivary gland with distinctive histomorphological features from that of MEC showed the same translocation t(11;19)(q21;p13), which is seen also in 60% of the MECs (10). Further translocations with alternative translocation partner such as t(11;17)(q22;p11) or t(11;13)(24q;q12) could be found in these tumors (11-16). The target genes in the t(11;19)(q21;p13) translocation are known (17). Molecular analysis of the translocation t(11;19)(q21;p13) identified a fusion transcript of the exon 1 of the mucoepidermoid carcinoma translocated-1 gene (*MECT1*, alias *CRTC1*, *TORC1*, *WAMTP1*) at 19p13 with the exons 2-5 of a novel member of the mastermind-like gene family (*MAML2*) at 11q21. The fusion transcript activates the transcription of the Notch target genes such as HES1 and HES5 (18,19). This fusion gene is also shared in lung mucoepidermoid carcinoma (20,21). Clear cell hidradenoma of the skin as the third tumor type with an identical *MECT1-MAML2* gene fusion was reported by Winnes *et al* (22) and Behboudi *et al* (23).

Materials and methods

Formalin-fixed, paraffin-embedded tumor samples. Nineteen tumor samples from 18 patients, diagnosed from 1988 to 2002 were retrieved from the archives of the Institute of Pathology Innsbruck, Austria and of the Institute of Pathology, Salzburg, Austria. Immunohistochemical examination was done with

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MIB-1 antibody (Dako, Austria, dilution 1:100, autoclave 1 bar, in citrate buffer for 30 min) using an automated immunostainer (Nexes, Ventana, Tuscon, AZ, USA). The evaluation of MIB-1 expression was determined as the percentage <10% and >10% of stained cells.

Comparative genomic hybridization (CGH). To evaluate if tumors have other abnormalities except 11q-aberrations, genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor material using standard protocols. Control DNA was prepared similarly from peripheral blood specimens of healthy individuals. Tumor (1 μg) and control (1 μg) DNAs were labeled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively by nick translation (Roche Diagnostics, Mannheim, Germany). After co-precipitation with 40 μg human Cot-1 DNA (Roche Diagnostics) and pre-annealing to suppress signals from repeated sequences the hybridization was carried out to normal human metaphase cells for 3 days at 37˚C. For detection the slides were stained with avidin-fluorescein isothiocyanate (Vector Labs Burlingame, CA) and anti-digoxigenin-rhodamine (Roche Diagnostics). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole and specimens were mounted in antifade solution (Vectashield, Vector Laboratories). Image acquisition was carried out using a fluorescence microscope (Zeiss Axioplan) equipped with a CCD camera (JAI M300) and ISIS software (Metasystems, Altlussheim, Germany). Gains or losses were calculated as significant by the evaluation software when fluorescence ratio values were <0.8 and >1.25. Pericentromeric, heterochromatic, telomeric regions and chromosome Y were excluded from the evaluation.

Identification of t(11;19) by fluorescence in situ hybridization (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of MECT1-MAML2 fusion transcript. FISH analysis. Sections (2 μm) were made from paraffin-embedded tissue blocks. The slide and probe preparations were performed according to the protocol for paraffin specimens of the manufacturer (Vysis Downers Grove, IL, USA) with minor modifications. Briefly, the slides were deparaffinized with three 10-min xylene washes, dehydrated in two 5-min washes in 100% ethanol. Subsequently, the slides were incubated in 10 mM citric acid buffer for 60 min at 80˚C, followed by an incubation in a pepsin solution (0.5 mg/ml), fixed in 4% formaldehyde for 10 min and dehydrated in a ethanol series (70, 90, 100%). To evaluate the CGH results, probes for centromere 2, 7, 16, 17, X, Y, LSI probes for 8q24, 9p21, 13q14, 20q13 and 22q11.2 as well as telomeric probes for 19p and 19q (Vysis) were applied onto the slides in the area of interest. Also a dual break apart probe (ZytoLight MEC I, ZytoVysion, Germany), a mixture of two clone contigs hybridizing to the chromosomal band 11q21 was used. The green-labeled probe (size ~550 kb) hybridizes proximal the *MAML2* gene, the orange-labeled probe (size ~400 kb) distal to *MAML2*.

The slides were covered with a glass cover slip, sealed with rubber cement, placed in the HYBrite system (Vysis) and denatured at 80˚C for 5 min and hybridized overnight at 37˚C. After two wash steps the slides were counterstained with DAPI in antifade solution. At least 165 nuclei (range 165-321, mean 216) were scored in each case with a fluorescence microscope, equipped with specific filters for SpectrumOrange, SpectrumGreen and DAPI. The images were acquired with a CCD camera and ISIS software.

RT-PCR analysis. Total RNA was extracted from 5-10 formalin-fixed, paraffin-embedded tissue sections (5 μm) from 19 MECs using High Pure RNA Paraffin Kit (Roche Diagnostics). Primers for RT-PCR for amplify the MECT1-MAML2 fragment with an expected size of 105 bp were *METC1* 5'-GCCTTCGAGGAGGTCATGA-3' and *MAML2* 5'-CTTGCTGTTGGCAGGAGA-3'. RT-PCR was run using a denaturation step at 94˚C for 3 min, followed by 35 cycles of 94˚C for 45 sec, 55˚C for 30 sec and 72˚C for 30 sec. A final extension step was done at 72˚C for 10 min. To check the quality of the cDNA it was also amplified in a GAPDH fragment with an expected size of 184 bp with the primers forward 5'-TCCATGACAACTTTGGTATC-3' and reverse 5'-TTCAGCTCAGGGATGACCTT-3'.

Results

Clinical and pathological summary. Seventeen samples were primary tumors and two were lymph node metastases. One of these lymph node metastases occurred in a patient with submandibular MEC within the first year after the initial surgical treatment (cases 9a and 9b, Table I). Distant metastases were not documented. Thirteen of the MECs originated from the major salivary glands with the majority located in the parotid gland and 3 cases derived from the submandibular gland. The remaining 2 tumors occurred in a minor salivary gland (soft palate). Tissue samples were stained with haematoxylin and eosin and classified by a pathologist according to the WHO classification (24). The age range at the time of diagnosis was 27-85 years with a median age of 59.4 years. Twelve of the 18 patients (66.7%) were >50 years of age. The male to female ratio was 1:1.6.

All patients presented one or more signs of symptoms. Clinical presentations were relatively uniform. The first symptom in all cases was non-inflammatory, painless solid tumefaction in the area of a salivary gland. Three parotid tumefactions were associated with facial palsy. Two patients experienced discomfort in the parotid region. Six patients (37.5%) had clinically positive cervical lymph nodes at the time of their examination; in all cases cervical metastases were confirmed by neck dissection. No patient had any signs of distant metastases at the time of diagnosis.

The tumors were clinically staged according to the TNM system (24). The sizes of the tumors ranged from 1.5 to 7 cm. Duration of symptoms ranged from 4 weeks to 18 months and did not correlate with tumor site or size. All patients were primarily treated with surgery. For tumors of the parotid gland a superficial (4 cases) or total (9 cases) parotidectomy was performed, supplemented by a suprahomohyoid (12 cases) or radical neck dissection (in 1 case). The 3 MECs of the submandibular gland were treated with suprahomohyoid neck dissection. Surgical management of MECs of minor salivary gland (2 cases) involved the local excision with suprahomohyoid neck dissection. All surgeries

Table I. Summary of clinical, histopathological and immunohistochemical data of 19 tumor samples from 18 patients with MEC.

Pat. no.	Gender/ Age	Tumor	S	G	TNM	Tumor localization/ tumor size (cm)	MIB-1 Expression	
							<10%	>10%
1	M/31	PT	6	2	pT2N0	Gl. p.r./2.5	+	
2	F/84	PT	3	3	pT3V1N1	Gl. p.r./4.8		+
3	M/42	PT	3	2	pT1N0	Gl. p.r./1.8	+	
4	M/44	PT	3	2	pT2N0	Gl. p.r./3	+	
5	M/73	PT	5	2	pT1N2b	Gl. p.l./2		+
6	M/47	PT	5	1	T1N0	Minor salivary gland /1.5	+	
7	F/65	LNM	5	3	N2bM0	Gl. p./7[a]		+
8	F/60	PT	5	1	T1N0	Gl. p.l./1.5	+	
9a	M/68	PT	3	3	T2N2b	Gl. subm.r./3.5		+
9b		LNM				Gl. subm.		+
10	F/54	PT	5	3	pT3N2b	Gl. p.l./5.5		+
11	F/82	PT	10	3	T4N1	Gl. p./6.5		+
12	F/69	PT	10	1	T2N0M0	Gl. p.l./3	+	
13	F/64	PT	10	3	pT2N0MX	Gl. subm./2.5		+
14	F/32	PT	10	1	T1N0	Gl. p.l./2	NA	
15	F/85	PT	5	3	pT3bN0MX	Gl. p./4.5		+
16	F/27	PT	5	3	pT2N0MXR1	Gl. p.r./3.2	+	
17	M/82	PT	5	2	pT2N1	Minor salivary gland/2.5	+	
18	F/61	PT	10	2	T2N0M0	Gl. subm./2.5	+	

Pat. no, patient number; M, male; F, female; PT, primary tumor; LNM, lymph node metastases; S, overall disease survival (years); G, tumor grading (G1, low grade, G2 intermediate, G3 high grade); Gl. p.r and Gl. p.l., Glandula parotis right and Glandula parotis left; Gl. subm., Glandula submandibularis; neck r, neck right; [a]size of the primary tumor; NA, not analysed.

were performed as curative resections, with all cases considered histopathologically to have tumor-free margins. The surgical margins were defined as negative in cases showing a rim of normal tissue of >3 mm around the tumor. Surgical treatment was supplemented with radiotherapy in 7 cases. The interval between surgery and the start of radiation therapy was 12-28 days. The radiotherapy was delivered using Co-60. Doses ranged from 54 to 70 Gy and the duration of therapy ranged from 35 to 42 days.

Follow-up ranged from 4 to 12 years. Of the 18 patients with MECs, 14 (77.8%) were alive without disease, 2 patients died from other causes (11%) and 2 patients died from their disease (11%). Four patients were free of the disease for 3 years; 8 patients for 5 years, 1 patient for more than 5 years. Five patients lived for >10 years and one of them died from another cause. Three patients developed local recurrence 23, 44 and 67 months postoperatively. Each recurrence (case no. 1, 8, 11) appeared in tumors of the parotid gland. The 5-year overall disease specific survival rate of all patients was 78%. Fifty percent of our tumors (9 cases) showed MIB-1 expression >10%. A summary of clinical and histopathological data is given in Table I.

CGH data. Fifteen primary MECs and 2 lymph node metastasis with sufficient DNA were analyzed with CGH. Copy number alterations were found in 13 of the analyzed 17 MECs (76.5%). In total, we detected 35 losses vs. 27 gains (1:1.29) with an average of 1.59 gains and 2.06 losses per tumor. Three of the 17 cases (17.6%) showed one aberration, 5.9% of the cases one (1/17) and 52.9% of the case (9/17) three or more aberrations. As the most frequent deviations gain on X (29.4%), partial or complete losses on chromosome 1, 2 and 15 (23.5%) were detected, followed by gain of 7/7p, losses of 17pq, 19/19p and 20/20p/20q in 17.6% (Table II; Fig. 1).

FISH data. FISH analysis was performed in all 19 tumors. In 7 of 19 tumors (36.8%) a *MAML2*-rearrangement at 11q21 was detected (Table I). As the most frequent, a signal pattern of one orange/green (yellow) signal (representing a normal 11q21 locus and a separate orange and green signal, demonstrating a disrupted 11q21 region) was seen (Fig. 3). This signal pattern suggests a t(11;19) or a variant of it, involving the 11q21 region. The other frequent chromosomal aberrations detected by CGH were confirmed by FISH.

RT-PCR results. The following RT-PCR-analysis showed the presence of a MECT1-MAML2 fusion transcript in 11 cases (57.9%), including the 7 FISH-positive cases (Fig. 2, not all data shown). A summary of our results is given in Table II.

Table II. Summary of CGH, FISH and RT-PCR results.

Pat. no.	CGH		FISH with 11q21 break apart probe	RT-PCR
	Losses	Gains	MAML2-Rearrangement (% aberrant cells)	Presence of MECT1-MAML2 fusion transcript
1	1pter-p32, 6pter-p21, 15q22-qter, 17, 20q, 22	18p, X	Yes (46)	Yes
2	15q22-qter	14	No	Yes
3	No imbalances	X	Yes (23)	Yes
4	No imbalances	X	Yes (57)	Yes
5	8pter-p21.1, 15	X	No	No
6	No imbalances	No imbalances	No	No
7	9q, 16, 17pter-q22, 18q	2q32.1-q34, 4q26-qter, 5q14-q23.3, 8q	No	No
8	No imbalances	No imbalances	Yes (50)	Yes
9a	No imbalances	19p	No	No
9b	5q11-q23, 14q11-q23, 17pter-q23, 18q,19q	X[a], 3q26-qter, 6q22	No	No
10	No imbalances	No imbalances	No	No
11	1pter-p32. 2[a], 6pter-p21, 10q23-qter, 14q22-qter, 15q22-qter, 22	4, 7, 9p, 13q14-q31, 18	No	Yes
12	No imbalances	No imbalances	Yes (84)	Yes
13	3p, 8p	5p, 7p, 20p	No	Yes
14	1[a], 2q[a]	20q	No	No
15	2q, 3p21.1-p12, 4[a], 13	19	No	Yes
16	1p31.1-p22.1, 2[a]	7, 19, 20	Yes (66)	Yes
17	NA	NA	No	No
18	NA	NA	Yes (56)	Yes

Pat. no, number of patients; [a]imbalances with a clear shift, but the CGH profile shows only a partially significant imbalance (partially reaching the threshold); NA, not analysed.

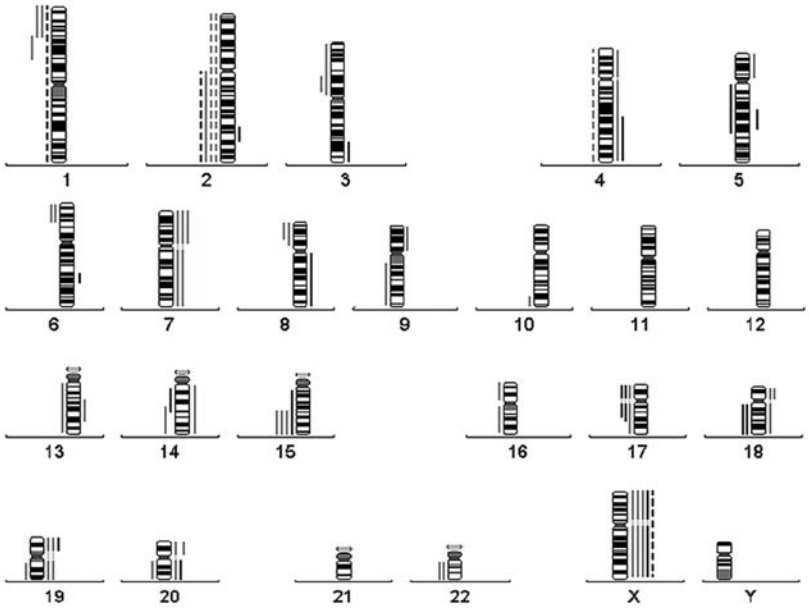

Figure 1. CGH results as summary profile for 17 analyzed MECs. Lines to the left side of the ideograms represent losses, lines to the right side chromosomal gains. Dotted lines indicate imbalances not reaching the diagnostic thresholds; gray lines indicate aberrations deriving from MECT1-MALM2 fusion transcript-positive cases and black lines aberrations from fusion transcript-negative cases.

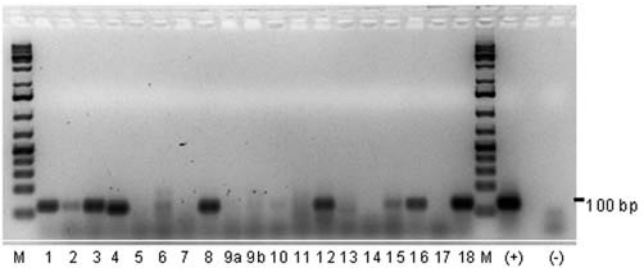

Figure 2. RT-PCR product of MECT1-MAML2 transcript in 19 formalin-fixed, paraffin-embedded tumors of 18 patients (not all data shown). M, marker; (+), positive control; (-), negative control.

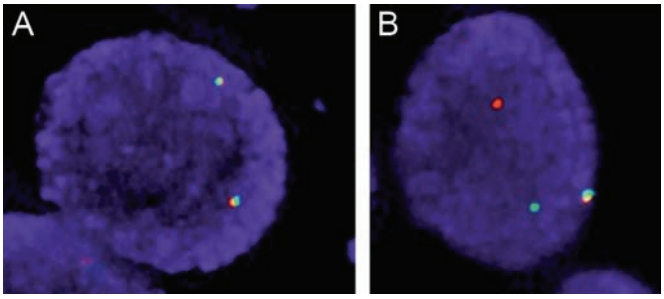

Figure 3. Interphase fluorescence *in situ* hybridization with the dual color, break apart probe MEC I represents MAML2 rearrangement. (A) Nucleus with two orange/green (yellow) signals indicating two normal 11q21 loci (case 2, Table I). (B) Nucleus with one orange/green (yellow) signal and a separate orange and green signal, caused by a break in 11q21 (case 8, Table I). This signal pattern was the most frequent finding in all analyzed cases.

The two small tumor groups with and without 11q-abnormalities did not allow a statistical analysis. Table III shows the association between the clinicopathologic parameters of patients with and without the presence of the 11q21 aberration.

Discussion

The presence of aberrations like translocation t(11;19)(q21;p13) is of special importance for future investigations, providing prognostic and therapeutic relevance. The impact of the METC1-MAML2 fusion on the clinical outcome in MECs is unclear und controversial in the literature. Our data give rise to several commentaries in comparison to the data by other authors:

i) In the present MECs 36.8% of the tumor samples showed 11q21 rearrangement. The RT-PCR analyzed tumors showed a MECT1-MAML2 fusion product in 57.9% of the tumors. This frequency is in agreement with studies on MECs by other authors (11,19). Several authors concluded that fusion positive tumors are biologically less aggressive with better clinicopathological behavior in comparison with fusion transcript negative tumors (11,20,32,33).

Behboudi *et al* (11) demonstrated that patients with fusion gene-positive tumors were substantially younger at clinical presentation, showing a predominance of smaller, high-differentiated low-grade tumors. Fusion-positive patients had a significant lower risk of local recurrence, metastases and tumor-related death. According to the study by Serra *et al* (20) all analyzed pulmonary MECs with 11q21 rearrangement were low-grade tumors. In contrast to these

Table III. 11q21-rearrangement and expression of MECT1-MAML2 fusion transcript in comparison with clinicohistopathological and genetic data.

	MECT1-MAML2-fusion transcript positive (n=11)	MECT1-MAML2-fusion transcript negative (n=7)
Mean age (years) (n=18)	59 (n=11)	60 (n=7)
Mean tumor size (n=18)	3.3 cm (n=11)	3.4 (n=7)
MIB-1 expression (n=18)		
<10%	7 (64%)	2 (29%)
>10%	4 (36%)	5 (71%)
Lymph node metastasis (n=18)		
Present	2 (18%)	5 (71%)
Absent	9 (82%)	2 (29%)
Tumor grade (n=18)		
G I, GII	6	4
G III	5	3
Mean survival time (years) (n=18)	6.36 (n=11)	5.43 (n=7)
No. aberrant CGH-cases	(n=8/10, 80%)	(n=5/7, 714%)
Average gains/aberrant case	2.0	2.2
Average losses/aberrant case	3.25	1.8
Aberrant cases with >2 CNV	5 (62.5%)	4 (80%)

authors in the cohort of Tirado and co-workers (33) the fusion transcript *METC1-MAML2* showed no association with tumor grade. The lacking of the fusion transcript was significantly associated with metastasis, suggesting that fusion-negative tumors represent a group of biologically aggressive tumors.

However, our study size is too small for a meaningful statistical consideration, but our fusion-negative tumors are predominately associated with occurrence of regional metastases (71% vs. 18%). In comparison to the study of Behboudi *et al* (11) the estimated median survival for fusion-positive patients was greater than in fusion-negative patients (10 years vs. 1.6 years). The mean survival time in patients with fusion-gene positive tumors in our collective was higher than in patients with fusions-negative tumors (6.36 years vs. 5.43 years). Regarding patient age, tumor grading and tumor size no difference could be observed between the fusion-negative and fusion-positive tumors.

ii) MIB-1 expression >10% we found predominately (87.5%) in high grade tumors (G3), whereas the most (89%) of the low to intermediate tumors (G1 to G2) were associated with MIB-1 expression <10%. Published immunohistochemical studies demonstrated also that high Ki-67 expression is significantly correlated with higher grade tumors (11,34,35). Regarding the fusion status we found more frequent MIB-1 expression <10% in fusion transcript-positive tumors than in MECT1-MAML2-negative tumors (77.8 vs. 22.2%). The part of our samples with expression greater than 10% was slightly lower in fusion-positive tumors (44.4 vs. 55.6%). In addition to our findings, the status of Ki-67-expression supplies additional prognostic information regarding tumor behavior.

iii) Extensive and systematic molecular genetic data for malignant tumors of the salivary gland have not been previously reported. Previous cytogenetic studies (by G-banding, SKY, FISH) of MECs of the salivary gland reported other, besides the common t(11;19), aberrations like -Y, +5, +7, +8, -14, +X and translocations involving chromosome 1, 3, 5, 6, 8, 12, 13, 15, 16 and 20 (11,15,21,25-30). To our knowledge, only two MEC cell lines with CGH data are available in the literature (21). In the first cell line gains or amplifications on 1q31, 5p, 6p22, 7pter-p15, 8q21.3-qter, 11p13-qter, 15q25-qter and losses of chromosome 9 and 20 were found. Frequent aberrations in the second analyzed cell line were gains at 3pter-p24, 3q, 7pter-q11.2 and 20. The regions 3p21-p13, 4p16, 4q32-qter, 5q32-qter and 8pter-p12 were under-represented.

The most frequent findings in our tumors were gains of X in 5 cases, partial or complete losses on chromosome 1, 2 and 15 in 4 cases, followed by gain of 7/7p, 19/19p and 20/20p/20q. MECs are characterized by a t(11;19)(q21;p13) translocation, more often occurring as sole anomaly being a simple translocation or complex one (6,31). The findings are only in partial concordance with the data by Tonon *et al* (21) and Behboudi *et al* (23) regarding gain on chromosome 7, 20, X and loss of 8p and 15.

iv) Copy number variation (CNV) and presence of MCET1-MAML2 fusion transcript: patients with fusion gene-positive tumors showed higher CNV (5.25 vs. 4.0) in fusion-negative tumors; whereas MECT1-MAML2-positive tumors showed more losses than gains/case (3.25 vs. 2.0) the number of losses and gains/case was approximately equal (1.8 losses vs. 2.2 gains) in fusion transcript negative tumors. In 62.5% of the fusion-positive tumors we detected more than 2 CNV in comparison with 80% in the fusion-negative tumors. Regarding the CGH-aberration spectrum, losses of 6p, 8p, 22 and gain of chromosome 7 and 18 were found only in MECT1-MAML2-positive tumors.

We concluded that the presence of MECT1-MAMl2 fusion transcript in MECs may define, in view to clinical and pathological outcome, a subset of tumors with more favorable outcome. Our findings in MECT1-MAML2-transcipt-positive tumors as a subgroup of MECs with regard to a better clinicopathological outcome is in agreement with most other publications. It remains to be clarified, if the presence of this aberration represents a useful diagnostic marker for prognosis and prediction of the biological tumor behavior.

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IV

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Contribution to the work

- Planning and performance of all done work, except the FISH analyses (FISH was performed by Dipl. Biol. Norbert Drieschner)
- Analysis of the qRT-PCR data
- Statistical analysis of the data
- Composing of the publication

A Link Between the Expression of the Stem Cell Marker *HMGA2*, Grading, and the Fusion *CRTC1-MAML2* in Mucoepidermoid Carcinoma

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Recently, the concept of cancer stem cells and their expression of embryonic stem cell markers has gained considerable experimental support. In this study, we examined the expression of one such marker, the *high-mobility group AT-hook 2 gene (HMGA2)* mRNA, in 53 formalin-fixed, paraffin-embedded mucoepidermoid carcinomas (MEC) and four normal parotid tissues using quantitative real-time RT-PCR (qPCR). MECs are often characterized by the fusion gene *CRTC1-MAML2,* the detection of which is an important tool for the diagnosis and prognosis of MEC. For detection of the *CRTC1-MAML2* fusion transcript, we performed RT-PCR. The mean expression level of *HMGA2* was higher in fusion negative (302.8 $\pm$ 124.4; $n = 14$) than in positive tumors (67.3 $\pm$ 13.1; $n = 39$). Furthermore, the fusion-negative tumors were often high-grade tumors and the *HMGA2* expression level rose with the tumor grade (low: 43.7 $\pm$ 11.0, intermediate: 126.2 $\pm$ 28.3, and high: 271.2 $\pm$ 126.5). A significant difference was found in the *HMGA2* expression levels between the different grading groups (one-way ANOVA, $P = 0.04$) and among the fusion-negative and -positive tumors (*t*-test, $P = 0.05$), indicating that the expression level of *HMGA2* was closely linked to grading, the presence/absence of the *CRTC1-MAML2* fusion, and the tumor behavior of MECs. These findings offer further evidence for the theory that the MEC group comprises two subgroups: one group with the *CRTC1-MAML2* fusion, which is a group with a moderate aggressiveness and prognosis, and the other group lacking that fusion corresponding to an increased stemness, and thus, higher aggressiveness and worse prognosis.

INTRODUCTION

In malignant neoplasms, a strong association between the overexpression of *high mobility group AT-hook 2 gene (HMGA2)*, the malignant phenotype, and an adverse prognosis has been demonstrated for a variety of malignancies (Rommel et al., 1997; Rogalla et al., 1997, 1998; Sezer et al., 2000; Gross et al., 2003; Langelotz et al., 2003; Miyazawa et al., 2004; Sarhadi et al., 2006; Meyer et al., 2007a,b; Winkler et al., 2007; Belge et al., 2008).

An overexpression has also been described for a variety of benign tumors, such as tumors of the salivary gland (Schoenmakers et al., 1995; Kazmierczak et al., 1996). HMGA2 is a chromatin-associated nonhistone protein that binds through its AT binding motifs to the minor groove of AT-rich DNA strands and acts as an architectural transcription factor (Reeves and Nissen, 1990; Bustin and Reeves, 1996; Zhou and Chada, 1998). The *HMGA2* gene is expressed predominantly during embryogenesis but is normally repressed in differentiated cells and tissues (Chiappetta et al., 1996; Rogalla et al., 1996; Hirning-Folz et al., 1998).

In this study, the expression of *HMGA2* in mucoepidermoid carcinomas (MECs) of either of two genetic types was compared with the expression in normal salivary gland tissues. MECs are the most frequent malignant tumors of the salivary glands (Goode and El Naggar, 2005). The histopathologic grade of MECs is an established

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predictor of prognosis and influences the treatment of the disease (Nance et al., 2008). A frequent subgroup of MECs is characterized by a recurring t(11;19)(q21;p13) translocation that has also been found in rare cases of Warthińs tumors and clear cell hidradenoma, which is often the sole cytogenetic alteration in this diseases (Enlund et al., 2004; Behboudi et al., 2006; El-Naggar, 2006; Winnes et al., 2007; Fehr et al., 2008a). Recent cloning of the translocation breakpoint in MEC cell lines identified a fusion gene comprised of exon 1 of the *CREB-regulated transcriptional coactivator CRTC1* (also known as *MECT1*, *TORC1*, or *WAMTP1*) gene at 19p13 and exon 2–5 of the *mastermind-like gene MAML2* at 11q21 (Tonon et al., 2003; Enlund et al., 2004). Previous functional studies have shown that the expression of *CRTC1-MAML2* is essential for the growth of translocation t(11;19)-positive MEC cell lines and that the N-terminal CREB-binding domain of CRTC1 is fundamental for the transforming activity of the fusion protein (Coxon et al., 2005; Wu et al., 2005; Komiya et al., 2006). Also, the fusion transcript *CRTC1-MAML2* has been linked with low- or intermediate-grade tumors and a good prognosis (Behboudi et al., 2006; Okabe et al., 2006). Notably, the t(11;19) and the underlying *CRTC1-MAML2* fusion are not restricted to MECs of the salivary glands but have been found in MECs of other organs (Stenman et al., 1998; Liu and Adams, 2007; Tirado et al., 2007), as well as in Warthin's tumors and clear cell hidradenoma (Bullerdiek et al., 1988; Enlund et al., 2004; Behboudi et al., 2005; Winnes et al., 2007; Fehr et al., 2008a). Furthermore, a variant of the *CRTC1-MAML2* fusion, *CRTC3-MAML2*, has been described (Fehr et al., 2008b).

HMGA2 is a gene abundantly expressed in embryonic stem cells. In malignant tumors, its reappearance has been linked to a more aggressive behavior, epithelial to mesenchymal transitions, and increased stemness of the cancer cell population. Accordingly, high expression of *HMGA2* usually correlates with a worse prognosis. Translocations affecting the HMGA2 locus also have been described in pleomorphic adenomas (PAs) of the salivary glands (Schoenmakers et al., 1995; Geurts et al., 1997, 1998), and amplifications of the gene can be found in subgroups of those tumors as well (Roijer et al., 1999, 2002). Persson et al. (2009) have shown that *HMGA2* may be important for the malignant transformation of PA to carcinoma ex PA (Ca-ex-PA).

The aim of this study was to analyze the *HMGA2* mRNA expression level in positive and negative MECs to find out if there was a correlation between expression level, tumor grade, and the presence/absence of the *CRTC1-MAML2* fusion transcript.

MATERIALS AND METHODS

Tumor Material

For this study, 53 formalin-fixed, paraffin-embedded (FFPE) MEC tissues were obtained from the Salivary Gland Registry of Oral Pathology at the University Medical Center Hamburg-Eppendorf and the Albertinen-Pathology, Hamburg, Germany. All tumors were carefully re-examined and the diagnosis of MEC confirmed according to the criteria of the World Health Organization (Goode and El Naggar, 2005). Four snap-frozen normal parotid tissues were used as a calibrator for the qPCR, and a MEC cell line (NCI-H292) positive for *CRTC1-MAML2* was used as a positive control for RT-PCR. For a summary of clinical and pathological data, see Table 1.

RNA Isolation

To validate the constant expression level of the endogenous control, total RNA was extracted from snap-frozen normal tissues with the TissueLyser and the RNeasy® Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). As the positive control for RT-PCR, RNA was isolated from NCI-H292 using TRIzol® LS reagent (Invitrogen, Karlsruhe, Germany). RNA was extracted from six 5 μm sections of FFPE tumors. The tissue was deparaffinated with xylene and the total RNA isolated with the High Pure RNA Paraffin Kit according to the manufacturer's instructions (Roche, Penzberg, Germany).

QPCR

cDNA synthesis of tumor and normal parotid RNA were performed with 150 ng of random hexamer primer, 250 ng of total RNA, 40 U of RNaseOUT™, and 200 U of M-MLV Reverse Transcriptase™ (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Quantitative real-time PCR amplifications were performed with the ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Darmstadt,

TABLE 1. Summary of Clinical and Histopathologic Data, *CRTC1-MAML2* Status, and *HMGA2* Expression in 53 Mucoepidermoid Carcinomas

Case no.	Age (years)	Sex	Histologic grade	*CRTC1-MAML2*[a]	RQ[b]
1	60	F	Low	+	199.0
2	80	F	Low	+	139.7
3	52	M	Low	+	85.4
4	71	M	High	+	22.6
5	72	F	Low	+	7.0
6	77	F	Low	+	7.6
7	79	F	Intermediate	+	83.3
8	74	F	Low	+	20.8
9	49	F	Low	−	57.6
10	63	M	High	−	1273.4
11	17	M	Low	+	7.3
12	79	F	Intermediate	+	149.0
13	38	M	Low	+	10.9
14	72	M	Intermediate	+	337.7
15	42	M	Low	+	41.8
16	42	M	High	−	66.2
17	75	F	Low	+	2.9
18	49	F	Intermediate	+	55.9
19	15	M	Low	−	24.9
20	65	M	High	−	1343.0
21	58	F	Low	+	36.3
22	40	M	Low	+	3.2
23	25	F	Intermediate	−	44.4
24	61	F	Low	+	25.1
25	62	M	Intermediate	+	81.2
26	47	M	Low	+	3.9
27	48	F	Low	−	88.5
28	37	M	Intermediate	−	101.8
29	61	F	Low	+	67.4
30	54	M	Intermediate	−	36.1
31	55	M	High	−	16.4
32	65	M	Intermediate	−	402.4
33	46	M	High	+	60.1
34	51	M	High	+	47.0
35	45	M	Low	+	7.8
36	34	F	Intermediate	+	140.0
37	45	M	Intermediate	+	6.3
38	75	F	Intermediate	+	36.4
39	76	F	High	+	24.9
40	65	M	High	+	139.4
41	71	F	Intermediate	+	17.8
42	–	M	High	−	693.0
43	49	M	High	−	37.1
44	44	M	High	+	15.2
45	49	F	Intermediate	+	281.8
46	58	F	Intermediate	+	256.1
47	62	M	Low	+	44.4
48	22	M	Intermediate	+	41.4
49	29	F	Intermediate	+	74.0
50	69	M	Intermediate	+	5.6
51	73	F	High	+	4.1
52	66	M	Low	+	35.1
53	80	F	High	−	53.8

[a]+, *CRTC1-MAML2*-positive; −. *CRTC1-MAML2*-negative.
[b]RQ, relative *HMGA2* expression level.

Germany). Because of different degradation of RNA isolated from formalin-fixed samples, the relative quantification method was used. In this study, 18S rRNA acted as an endogenous control (Antonov et al., 2005) and *HMGA2* as the target gene. The primer pair of the *HMGA2* assay (TaqMan® Assay no. Hs00171569_m1/Applied Biosystems) spans from exon 1 to 2 and amplifies a 65-bp fragment. *HMGA2* and 18S rRNA expression analyses were performed in triplicate in a total volume of 20 µl using 2 µl of each cDNA corresponding to 25 ng of total RNA for *HMGA2* and 2.5 ng for 18S rRNA quantification. For each PCR run, nontemplate controls and reactions without reverse transcriptase were included. We used tissue from four normal salivary glands to calculate the calibrator value (mean value of the probes). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles with 15 sec at 95°C and 1 min at 60°C. The relative expression levels of all probes were calculated using the ΔΔCt method and SDS Software v1.2.3 (Applied Biosystems).

RT-PCR

For cDNA synthesis of MEC RNA, 1 µg of total RNA was used for reverse transcription using 200 U of M-MLV Reverse Transcriptase™ with random primers according to the manual (Invitrogen, Karlsruhe, Germany). For cDNA synthesis of PCR-positive control (NCI-H292), 2 µg of total RNA was reverse transcribed using 200 U of M-MLV Reverse Transcriptase™ (Invitrogen) according to the manufacturer's instructions, using a poly(A)-oligo$(dt)_{17}$ primer.

Primers for amplification of the *CRTC1-MAML2* fragment with an expected size of 105 bp were *CRTC1* 5′-GCCTTCGAGGAGGTCATGA-3′ and *MAML2* 5′-CTTGCTGTTGGCAGGAGA-3′. The PCR amplification of the cDNA was performed in a final volume of 50 µl containing 5 µl template cDNA, 5 µl 10× PCR buffer without Mg^{2+}, 0.5 µM of each primer, 200 µM dNTP mix, 1.5 mM $MgCl_2$, and 0.5 µl Taq DNA Polymerase (5 U/µl) (Invitrogen). RT-PCR was performed using a denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 30 sec and 72°C for 30 sec. A final extension step was carried out at 72°C for 10 min.

To check the quality of the cDNA, a *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* fragment with an expected size of 184 bp was also amplified with the forward primer 5′-

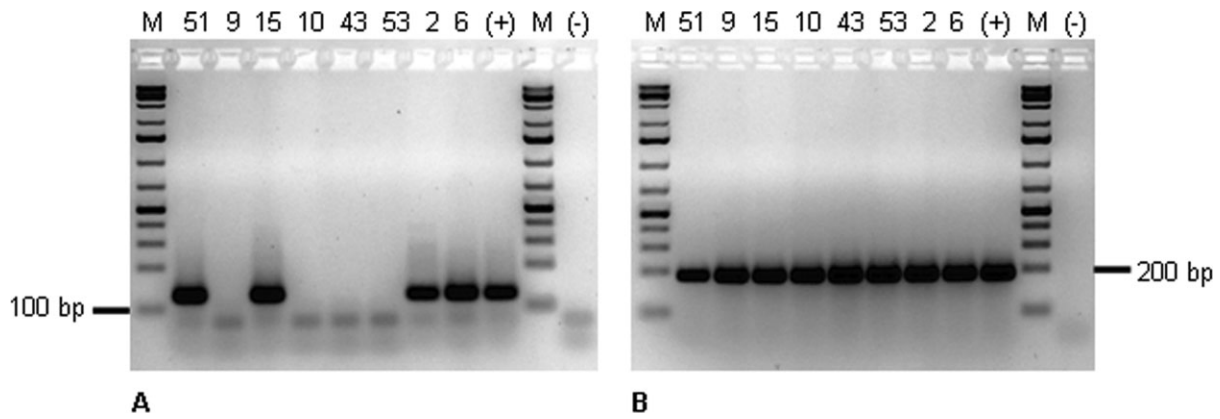

Figure 1. Results of RT-PCR on mucoepidermoid carcinomas (A) from *CRTC1-MAML2* PCR and (B) from *GAPDH* PCR; not all data shown; (+), PCR - positive control; (−), PCR - negative control.

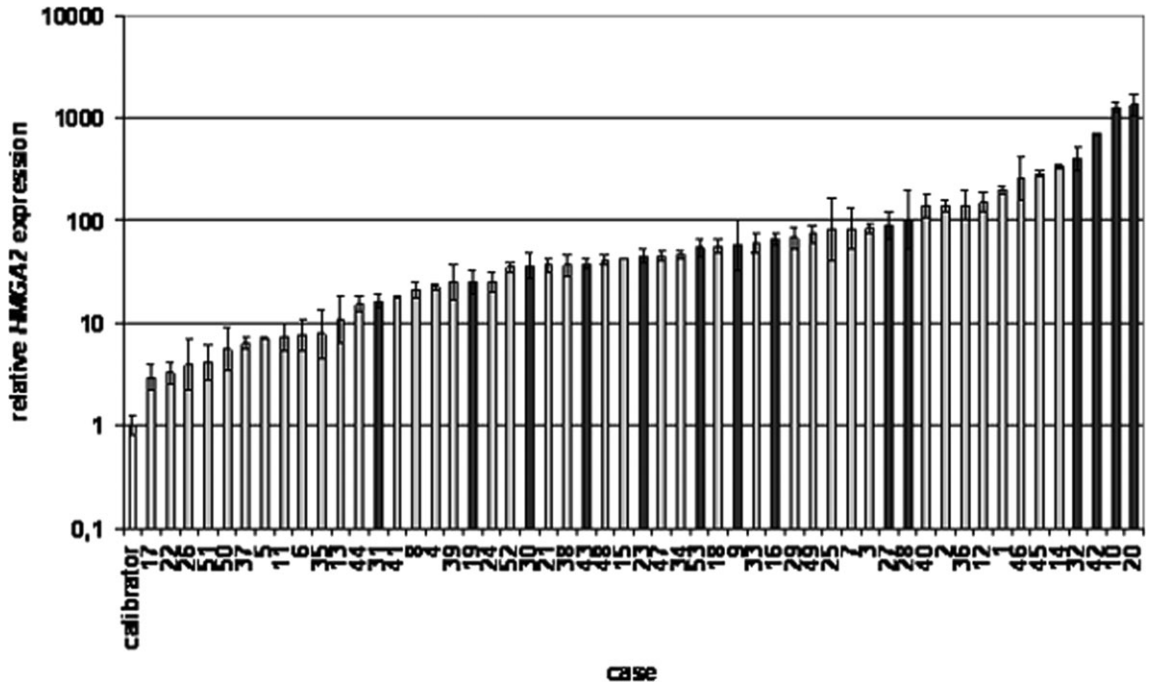

Figure 2. Results of relative *HMGA2* quantification in mucoepidermoid carcinomas. The mean value of three normal tissues serves as calibrator (white bar). Different *CRTC1-MAML2* results are represented by bar colors: dark gray: *CRTC1-MAML2* - negative, pale-gray: *CRTC1-MAML2* - positive.

TCCATGACAACTTTGGTATC-3′ and the reverse primer 5′-TTCAGCTCAGGGATGACCT T-3′ After an initial denaturation at 94°C for 3 min, PCR runs were performed over 35 cycles of amplification, using the following conditions: 94°C for 45 sec, 62°C for 45 sec, and 72°C for 30 sec; the run was completed with a final extension for 10 min at 72°C.

Statistical Analyses

Data were statistically analyzed with a one-tailed Student's *t*-test, two-sided Fisher's test, and one-way ANOVA test. A *P*-value of <0.05 was considered a statistically significant difference.

RESULTS

Detection of the *CRTC1-MAML2* Fusion Transcript in Archival Specimens

Of a total of 53 MECs, 74% ($n = 39$) showed evidence of a *CRTC1-MAML2* fusion. The fusion gene was mainly found in low-grade (46%) and intermediate-grade (36%) tumors; only 18% of the *CRTC1-MAML2*-positive samples were high-

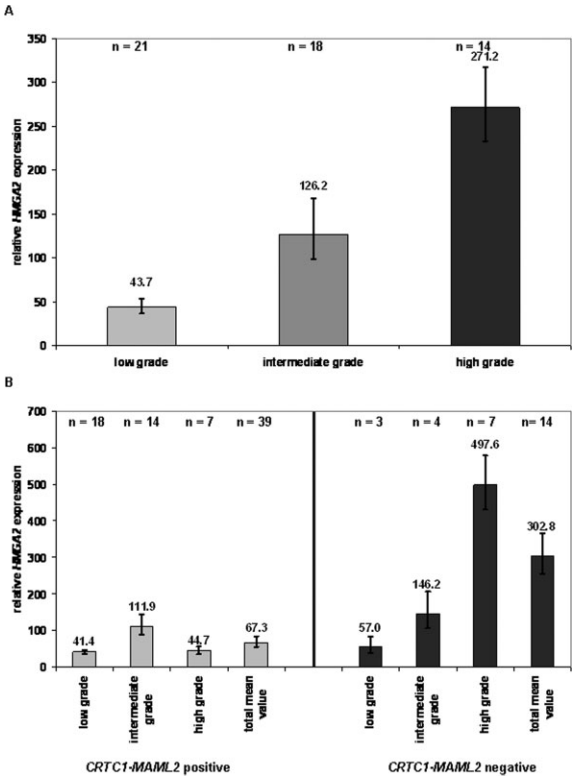

Figure 3. Mean values of *HMGA2* expression in all mucoepidermoid carcinomas (A) in relation to the grading and (B) in relation to the grade and the presence/absence of *CRTC1-MAML2*.

grade tumors. Three cases of 14 *CRTC1-MAML2*-negative samples were from low-grade (21%), four cases from intermediate-grade (29%), and seven cases from high-grade tumors (50%). The results of the *CRTC1-MAML2* RT-PCR are summarized in Table 1. Figure 1 shows the results of (A) the *CRTC1-MAML2* RT-PCR and (B) the *GAPDH* RT-PCR.

HMGA2 Expression in *CRTC1-MAML2*-Positive and -Negative Tumors

The expression of 18S rRNA from snap-frozen parotid tissue showed only low variation, with a mean Ct value of 8.12 ± 0.12. Therefore, 18S rRNA was chosen as the endogenous control for relative quantification. In all tumor samples, an increased expression of *HMGA2* was detected. The highest expression was found in a few fusion transcript-negative high-grade tumors (Fig. 2). In the total cohort of MECs, the mean *HMGA2* expression value rose with the tumor grade (low: 43.7 ± 11.0; intermediate: 126.2 ± 28.3; high: 271.2 ± 126.5) (Fig. 3A). Expression was higher in *CRTC1-MAML2*-negative (low: 57 ± 18.4; intermediate: 146.2 ± 86.7; high: 497.6 ± 227.9) than in fusion-positive tumors (low: 41.4 ± 12.5; intermediate: 111.9 ± 28.8; high: 44.7 ± 17.3) (Fig. 3B).

HMGA2 expression differences between the groups were analyzed using one-tailed Student's *t*-test (Table 2) and one-way ANOVA. One-way ANOVA revealed a significant difference between the *HMGA2* medians of the three grading stages ($P = 0.04$).

TABLE 2. Results of Student's *t*-Test Analyses of *HMGA2* Expression Levels

Parameter	*P*-value
All probes	
Normal tissue vs. total cohort of MEC	$P = 0.0004$
Fusion genes	
Normal tissue vs. *CRTC1-MAML2*-positive	$P \leq 0.0001$
Normal tissue *vs.* *CRTC1-MAML2*-negative	$P = 0.02$
CRTC1-MAML2-positive vs. *CRTC1-MAML2*-negative	$P = 0.05$
Histologic grade	
High-grade (*CRTC1-MAML2*-positive) vs. High-grade (*CRTC1-MAML2*-negative)	$P = 0.04$

TABLE 3. Clinicopathologic Correlations with *CRTC1-MAML2* Fusion Transcript Status in MECs

Parameter	Positive ($n = 39$)	Negative ($n = 13/14$)	*P*-value
Age			
>40	5	3	$P = 0.40$
<40	34	10	
Gender			
Male	19	10	$P = 0.20$
Female	20	4	
Grading			
Low/Intermediate	32	7	$P = 0.03$
High	7	7	

Clinicopathologic Correlation of *CRTC1-MAML2* Fusion Transcript in MECs

Group results were analyzed using a two-sided Fisher's test (Table 3). Although no correlation among gender, age, and fusion transcript status was evident, a correlation between the grading stages low/intermediate and high was found ($P = 0.03$).

DISCUSSION

The Mitelman Database lists a large group of a variety of tumors with alterations of the chromosomal region 12q13-15 (Mitelman Database of Chromosome Aberrations in Cancer, 2009), including a group of salivary gland tumors

(Stenman and Mark, 1983; Bullerdiek et al., 1987, 1993; Wanschura et al., 1995; Kasamatsu et al., 2005; Rutherford et al., 2005). These alterations commonly result in deregulation of *HMGA2*-containing oncogenes (Schoenmakers et al., 1995; Geurts et al., 1997, 1998; Persson et al., 2009). Several authors have described a strong association between the overexpression of *HMGA2*, the malignant phenotype, and an adverse prognosis of carcinomas (Rommel et al., 1997; Rogalla et al., 1997, 1998; Sezer et al., 2000; Gross et al., 2003; Langelotz et al., 2003; Miyazawa et al., 2004; Sarhadi et al., 2006; Meyer et al., 2007a,b; Winkler et al., 2007; Belge et al., 2008).

In this study, the *HMGA2* mRNA expression in two different subtypes of MECs was analyzed to examine possible correlations between expression level, tumor grade, and the presence/absence of the *CRTC1-MAML2* fusion. We quantified the *HMGA2* expression level in 53 MECs of the two types by qPCR. The results of qPCR showed highly significant differences in the expression level of *HMGA2* between the calibrator (median of four normal tissues) and the tumor tissues ($P = 0.0004$). Furthermore, there was a significantly higher level of *HMGA2* expression in high-grade tumors when compared with low-grade tumors.

The *CRTC1-MAML2* fusion gene was significantly more often detected in low-/intermediate-grade tumors than in tumor tissues of high grade. We also noted a lack of correlation among gender ($P = 0.2$), age ($P = 0.4$), and fusion transcript status. Previous studies reported similar findings (Okabe et al., 2006; Tirado et al., 2007). Behboudi et al. (2006) showed that the clinical prognosis for low-grade MECs of the salivary glands was much better than for high-grade tumors, and Tirado et al. (2007) described *CRTC1-MAML2*-negative tumors to be more likely to develop distant metastases. These observations were confirmed by our results: *CRTC1-MAML2*-negative tumors were frequently high-grade tumors (7 of 14 cases, 50%), and the *HMGA2* level was significantly higher in *CRTC1-MAML2*-negative tumors than in *CRTC1-MAML2*-positive tumors.

The results of this study confirmed that the *CRTC1-MAML2* transcript was present in the majority of MECs. These results correlate well with previously reported studies (Martins et al., 2004; Behboudi et al., 2006; Tirado et al., 2007; Fehr et al., 2008b). The *CRTC1-MAML2*-positive tumors in this study frequently had a low or intermediate grade (32 of 39 cases, 82%), whereas positive high-grade tumors were relatively rare (7 of 39 cases, 18%); these results correlate with previously reported results (Tirado et al., 2007; Fehr et al., 2008b).

The one-way ANOVA test revealed a significant variation between the *HMGA2* medians of the three grading stages. The histopathologic grade of a MEC is an established predictor of prognosis and treatment. Auclair et al. (1992) found that the 5-year mortality rates for MECs were 3, 10, and 46% for low-, intermediate-, and high-grade tumors, respectively (Goode et al., 1998). Because of the more aggressive behavior of high-grade compared with low-grade MECs, a more intensive treatment has been recommended for the former group (Nance et al., 2008). In comparison to the calibrator, all examined tumors showed an increased *HMGA2* expression level of 3- to 1343-fold. Furthermore there was a correlation between the *HMGA2* expression level, the presence/absence of the *CRTC1-MAML2* fusion gene, and the grade of the tumor. These results indicated that the expression level of *HMGA2* was closely linked to grading and the *CRTC1-MAML2* fusion, as well as with the tumor behavior of MECs.

The general association between high *HMGA2* level and the aggressiveness of solid tumors could be an interesting starting point for a possible *HMGA2* silencing therapy; previous reports have discussed this form of therapy for thyroid neoplasms, liposarcomas, and ovarian cancer. The authors and others have silenced the *HMGA2* gene, both in vitro and in vivo, which results in growth inhibition of the tumor and increased apoptosis (Berlingieri et al., 1995; Pentimalli et al., 2003; Malek et al., 2008).

This leads to considerations on the correct classification of MECs. The present classification of MEC includes two subgroups: a large group with the translocation t(11;19) and the *CRTC1-MAML2* fusion (with a moderate aggressiveness and prognosis), and a smaller group lacking this fusion (more aggressive and with a worse prognosis). Previous functional studies have shown that the expression of *CRTC1-MAML2* is essential for the growth of t(11;19)-positive MEC cell lines and that the N-terminal CREB-binding domain is fundamental for the transforming activity of the chimeric protein (Coxon et al., 2005; Wu et al., 2005; Komiya et al., 2006). Like *Drosophila Mastermind* and *MAML1*, full-length *MAML2* acts as a CSL-dependent transcriptional coactivator of Notch (Wu et al., 2000, 2002; Lin et al., 2002) In contrast, CRTC1-MAML2 activates the

transcription of Notch target genes independent of Notch ligands and CSL binding sites. In addition, the recent identification of the CRTC1 protein as a potent coactivator for genes that are regulated by cAMP-responsive elements suggests that *CRTC1-MAML2* may disrupt both Notch and CREB signaling pathways and induce tumorigenesis (Conkright et al., 2003; Iourgenko et al., 2003; Wu et al., 2005). The second group, which lacks the *CRTC1-MAML2* fusion, may represent a distinct category of poorly differentiated carcinomas of non-MEC etiology (Behboudi et al., 2006; Okabe et al., 2006; Tirado et al., 2007) with aberrantly high *HMGA2* levels. These two groups might have different origins, with the subgroup characterized by a *CRTC1-MAML2* fusion representing a histogenetic link within Warthin's tumor (Bell et al., 2008). One possible origin for the second subgroup could be the translocation t(3;8)(p21;q12) (Bullerdiek et al., 1990) in a MEC. Interestingly, this translocation is typically found in PAs of the salivary glands (Mark and Dahlenfors, 1986; Mark et al., 1988; Fonseca et al., 2008), and Persson et al. (2009) have hypothesized that amplification of *HMGA2* may cause the malignant transformation of a benign PA into a Ca-ex-PA. However, for this study on MECs, we think that the elevated mRNA levels are as a rule due to a global dedifferentiation observed in the tumors rather than to mutations affecting HMGA2. This was suggested by the result of FISH analyses on a few of the tumors, with and without the *CRTC1-MAML2* fusion, using a HMGA2 break-apart probe. The FISH analysis showed neither amplification nor rearrangements of the HMGA2 locus (data not shown). However, we cannot rule out minor changes of the HMGA2 locus which the FISH break-apart probe cannot detect.

In summary, this study indicated that the MEC group comprises two subgroups. The first had the *CRTC1-MAML2* fusion that may affect the Notch and CREB pathways, and is a group with a moderate aggressiveness and prognosis. The second subgroup had aberrant *HMGA2* expression levels and has a higher aggressiveness and worse prognosis. This aberrant *HMGA2* expression level makes *HMGA2* an interesting therapeutic target against high-grade MECs.

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