Studies of Gene Fusions and Copy Number Alterations in Salivary and Adnexal Neoplasms

Marta Persson

UNIVERSITY OF GOTHENBURG

Sahlgrenska Cancer Center

Department of Pathology

Sahlgrenska Academy at University of Gothenburg

Sweden

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-To my lovely family

"Anyone who has never made a mistake
has never tried anything new"
-Albert Einstein

ABSTRACT

Studies of Gene Fusions and Copy Number Alterations in Salivary and Adnexal Neoplasms

Marta Persson

Sahlgrenska Cancer Center, Department of Pathology,
Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

Cancer is a genetic disease caused by the accumulation of genetic changes such as mutations and chromosomal rearrangements. An increasing number of genetic studies of both hematological and solid neoplasms have shown that recurrent chromosome translocations often result in fusion oncogenes. These are considered as early events in tumorigenesis and are often key regulators of cellular transformation. We have previously shown that the t(6;9)(q22-23;p23-24) translocation is a recurrent genetic alteration in adenoid cystic carcinoma (ACC) and that the recurrent t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma (MEC) results in a *CRTC1-MAML2* gene fusion. Here, we have used a combination of genetic and molecular techniques, including FISH, RT-PCR, qPCR, transfection studies, and arrayCGH, to (i) gain further insights into the molecular pathogenesis of *CRTC1-MAML2* positive/negative MECs and hidradenomas and to study the clinical significance of this fusion, (ii) to identify the target genes of the t(6;9) in ACC and to study the molecular consequences of this rearrangement, and (III) to characterize the genetic profile of ACC using high-resolution arrayCGH and to identify candidate target genes located within regions of copy number alterations (CNA).

Detailed analyses of 29 MECs revealed *CRTC1-MAML2* fusions in 55% of the tumors. The CRTC1-MAML2 fusion protein was expressed in all three MEC-specific cell types and co-localized with CREB in nuclear granules. Analyses of potential targets of the fusion revealed differential expression of cAMP/CREB and Notch targets in fusion-positive and -negative MECs respectively. Interestingly, fusion-positive patients had a significantly lower risk of local recurrence, metastases or tumor related death compared to fusion-negative patients ($p<0.001$), and the estimated median survival for fusion-positive patients was >10 years compared to 1.6 years for fusion-negative patients. Our findings suggest that MECs may be molecularly classified based on the presence or absence of the *CRTC1-MAML2* fusion and that the fusion is a useful marker in predicting the biological behavior of MECs.

Analyses of 20 benign cutaneous hidradenomas showed that the *CRTC1-MAML2* fusion is recurrent in the clear cell variant of this tumor. The results indicate that the fusion is etiologically linked to benign and low-grade malignant tumors originating from diverse exocrine glands.

Positional cloning of the t(6;9) translocation in ACC of the breast and head and neck revealed a new mechanism of activation of the *MYB* oncogene involving gene fusion. The fusion gene consists of *MYB* exons 1-14 fused to the last coding exon(s) of the transcription factor gene *NFIB*. The fusion results in loss of the 3´-end of *MYB*, including several conserved binding sites for miRNAs that regulate *MYB* expression negatively. The data indicate that deletion of these target sites may disrupt repression of *MYB* leading to overexpression of *MYB-NFIB* transcripts and protein and to activation of critical *MYB* target genes. Our findings also indicate that the *MYB-NFIB* fusion is a hallmark of ACCs and that deregulation of *MYB* and its target genes are key oncogenic events of both diagnostic and therapeutic significance in ACC. High-resolution arrayCGH analysis of 40 *MYB-NFIB* fusion-positive and -negative ACCs, revealed novel CNAs and significant refinements of previously detected CNAs. The most frequent alterations were losses involving 12q, 6q, 9p, 11q, 14q, 1p, and 5q and gains involving 1q, 9p and 22q. Using an integrated copy number and global gene expression approach, we identifed several candidate target genes, including *NBL1*, *SFN*, *PLAGL1*, and *NR4A1*, that were down-regulated in tumors with 1p, 6q or 12q deletions compared to tumors without these CNAs. Further characterization of these regions and genes may lead to identification of new biomarkers of pathogenetic, prognostic, and therapeutic importance for ACC.

Key words: chromosome translocation, fusion oncogene, *MYB*, *NFIB*, *CRTC1*, *MAML2*, salivary gland, breast, adenoid cystic carcinoma, mucoepidermoid carcinoma, hidradenoma

LIST OF PAPERS

This thesis is based on the following papers that will be referred to in the text by their roman numerals:

I. Behboudi A, Enlund F, **Winnes M**, Andren Y, Nordkvist A, Leivo I, Flaberg E, Szekely L, Makitie A, Grenman R, Mark J, Stenman G. Molecular classification of mucoepidermoid carcinomas-prognostic significance of the *MECT1-MAML2* fusion oncogene. *Genes Chromosomes Cancer* 2006;45:470-81.

II. **Winnes M**, Molne L, Suurkula M, Andren Y, Persson F, Enlund F, Stenman G. Frequent fusion of the *CRTC1* and *MAML2* genes in clear cell variants of cutaneous hidradenomas. *Genes Chromosomes Cancer* 2007;46:559-63.

III. **Persson M**, Andrén Y, Mark J, Horlings HM, Persson F, Stenman G. Recurrent fusion of *MYB* and *NFIB* transcription factor genes in carcinomas of the breast and head and neck. *Proc Natl Acad Sci U S A* 2009; 106:18740-4.

IV. **Persson M**, Moskaluk C, Frierson Jr HF, Persson F, Andrén Y, Nordkvist A, Stenman G. Combined genomic profiling and gene expression analysis reveal recurrent copy number alterations and candidate target genes in adenoid cystic carcinoma. Manuscript.

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INTRODUCTION

Cancer

Cancer is a genetic disease involving changes of the genome (Boveri, 1914; Bishop, 1987; Weinberg, 1989; Hanahan and Weinberg, 2000; Hahn and Weinberg, 2002; Vogelstein and Kinzler, 2004). It is a complex multistep process characterized by a series of genetic events which convert a normal cell into a neoplastic cell by liberating it from its normally so tightly governed proliferation and growth control mechanisms (Klein and Klein, 1985; Bishop, 1991; Weinberg, 1989). Genetic changes in cancer include gene mutations and structural and numerical chromosomal aberrations. It is the accumulation of such genomic changes in somatic cells, over a prolonged period of time, that provide these cells with an advantage against their surrounding cells and subsequently lead to tumor formation (Vogelstein and Kinzler, 1993; Hahn and Weinberg, 2002).

In order for normal cells to progress to neoplastic cells, a varying number of genetic alterations are needed, depending on the cell and tumor type (Hahn et al., 1999; Hanahan and Weinberg, 2000). Approximately 90% of all neoplasms are sporadic lesions that occur when a somatic cell has accumulated a sufficient number of genetic alterations for a tumor to develop. The remaining 10% are hereditary neoplasms. The latter cases are caused by predisposing mutations in germ line cells. In these cases the offspring carries the mutation in all cells in the body. Several tumor types may occur in both sporadic and hereditary forms.

Most, if not all, neoplasms are thought to be of monoclonal origin and therefore originate from a common ancestral cell (Knudson, 1985; Wainscoat and Fay, 1990). The accumulation of genetic changes that turns the cell "neoplastic" provides it at the same time with a selective growth advantage over adjacent normal cells (Novell, 1976) (Figure 1). As a result of genetic instability, cancer cells often acquire a variety of genetic changes that over time generate tumors consisting of an increasingly abnormal and heterogeneous tumor cell population (Nowel, 1976; Woodruff, 1983; Heppner, 1984). One important difference between benign and malignant tumors is that malignant tumors have acquired additional genetic changes making them capable of invading surrounding tissues and forming distant metastases (Yokota, 2000).

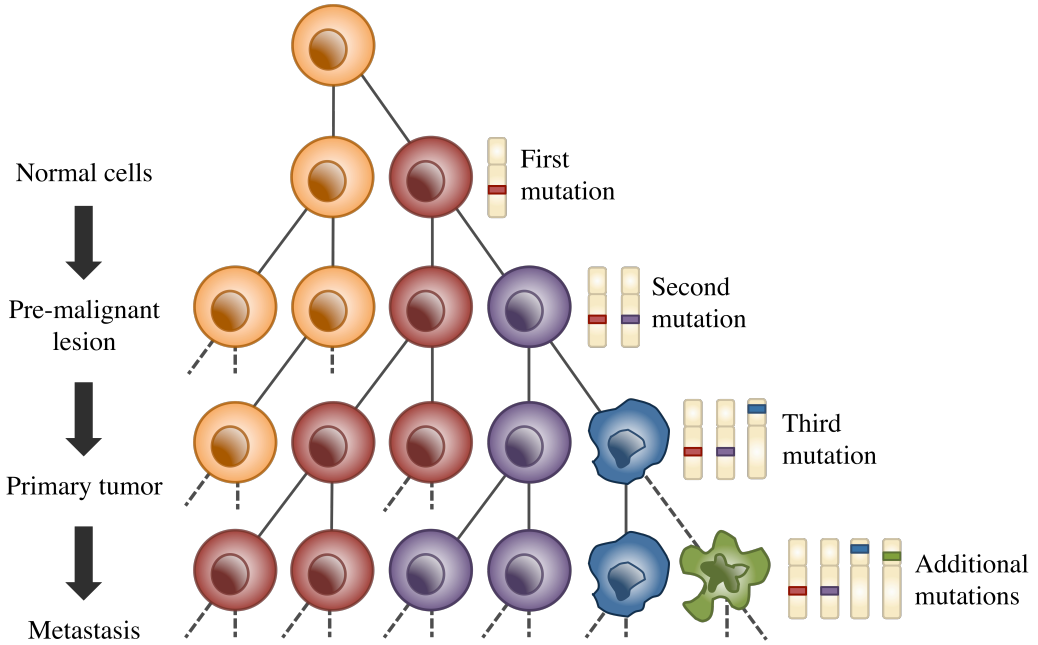

Figure 1. Cellular transformation is a multistep process where the accumulation of genetic alterations leads to tumor development and progression. *Modified from Cavenee et al., 1995.*

Oncogenes

The genome contains a large set of proto-oncogenes, whose functions are to regulate normal cell proliferation and differentiation. Oncogenes are dominant genes and gain-of-function mutations in one allele is therefore enough to disrupt and/or deregulate the expression of these genes and convert them into oncogenes. It is the activation and aberrant expression of oncogenes that contribute to abnormal cellproliferation and tumorigenesis (Bishop, 1991; Vogelstein and Kinzler, 2004). Several hundreds of oncogenes are known today, all discovered during the last three decades that have passed since the first oncogene was identified by Stehelin and co-workers in 1979. They discovered that the transforming potential of the Rous sarcoma virus in avians was due to a normal cellular gene, named *SRC* (Stehelin et al., 1976). It is now known that oncogenes can be activated through several different mechanisms in addition to viral transduction, such as point mutation, gene amplification and chromosomal rearrangements such as translocations, insertions and inversions (Bishop, 1991; Weinberg, 1994).

Examples of oncogenes activated by point mutations include members of the *RAS*-family. Overexpression of *RAS* enhances cell cycle progression and has been detected in a variety of human tumor types, including pancreatic carcinoma, lung cancer, colorectal cancer, and thyroid carcinoma (Bos, 1989; Macaluso et al., 2002). Oncogenes activated by gene amplification include *EGFR*, *MYCN* and *ERBB2*. *EGFR* is, for example, amplified in lung cancer and malignant gliomas

(Humphrey et al., 1988), *MYCN* in neuroblastomas (Schwab et al., 1983) and *ERBB2* in breast cancers (Slamon et al., 1989). Activation of oncogenes by chromosomal rearrangements, such as translocations, insertions and inversion, is a frequent event in numerous neoplasms, both solid tumors and hematological neoplasms. The most well-known oncogene activated by translocation is the ABL oncogene in chronic myeloid leukemia (CML). The CML-specific translocation t(9;22), also known as the Philadelphia chromosome, results in a fusion between the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 (Nowell and Hungerford, 1960; Rowley, 1973). The fusion protein is a constitutively activated tyrosine kinase and is found in virtually all patients with CML.

Activated oncogenes, irrespective of the activating mechanism, drive the neoplastic process by increasing the number of tumor cells by stimulating of mitosis and by inhibiting cell death. They function as growth factors (e.g. *PDGFB*), growth factor receptors (e.g. *EGFR*), signal transducers (e.g. *SRC* and the RAS-family), DNA-binding nuclear transcription factors (e.g. *MYC*, *JUN* and *FOS*) and cell cycle regulators (e.g. *CCND1* and *CDK4*) (Dolittle et al., 1983; Waterfield et al., 1983; Wells and Bishop, 1988; Brown and Cooper, 1996; Barbacid, 1987; Bos, 1989).

Tumor suppressor genes

Cell proliferation is regulated by growth-promoting proto-oncogenes, as mentioned above, and counter-balanced by growth-constraining tumor suppressor genes (TSG) or gatekeeper genes. Inactivating loss-of-function mutations in TSGs liberate the cell from the constraints imposed by theses genes and contribute to the uncontrolled growth behavior of cancer cells (Weinberg, 1991). There are three cardinal properties of "classic" TSGs. First, they are recessive in nature and must therefore undergo biallellic inactivation in tumor cells. This can be achieved by deletions, mutations or DNA methylation (Jones and Laired, 1999; Sherr, 2004; Weinberg, 2007). Second, the inheritance of one mutant allele accelerates tumor susceptibility, as demonstrated by Knudson in his "two hit" model (Knudson, 1973; Sherr, 2004). Third, inactivation of the same TSG may also occur in sporadic cancers (Sherr, 2004). Proteins encoded by TSGs are involved in cellular functions, such as transcriptional regulation, inhibition of proliferation, apoptosis and genetic stability. Examples of well-known TSGs are *RB1*, *TP53*, *WT1*, *APC*, *NF1*, *NF2*, *BRCA1* and *BRCA2*.

The first TSG to be molecularly cloned was the retinoblastoma gene *RB1* (Friend et al., 1986). The RB gene encodes a nuclear phosphoprotein, pRB1, which acts as a repressor of the cell cycle. The principal effect of the protein is to connect the cell cycle clock with the transcriptional control of cell cycle progression through the G_1 phase by interacting with transcription factors such as *EGF1*. The most

well-known TSG is perhaps *TP53*. The key functions of TP53 are mediation of cell cycle arrest and induction of apoptosis as a response to cellular stress, including irradiation and hypoxia, DNA-damage and even oncogenic activation (Levine 1993, 1997; Haffner and Oren, 1995; Prives, 1998). Inactivation of TP53 results in the accumulation of mutations and eventually in a genomic instability (Weinberg, 2007). TP53 is perhaps the most frequently mutated gene in human cancer and it is mutated in more than 50% of human cancers (Sherr, 2004). In addition to TP53, the CDKN2A/2B genes are also frequently altered in human cancers (Ruas and Peters, 1998). This locus encodes two different tumor suppressor proteins; $p16^{INK4A}$, which inhibits phosphorylation of pRB1, and $p14^{ARF}$, which stabilizes TP53 through direct interaction with MDM2 (Quelle, 1995; Sharpless and DePinho, 1999; Sherr, 2000, 2001, 2004). Taken together, these four TSGs (TP53, RB1 and CDKN2A/2B) constitute the foundation of a signaling network that monitors mitogenic signals and restrains abnormal growth-promoting signals from driving cell cycle progression in an uncontrolled manner. Inactivation of this network occurs in most, if not all, human cancers (Sherr, 2004).

DNA repair genes

The third class of cancer genes is DNA repair genes, also referred to as caretakers or stability genes, since their main function is to maintain the integrity of the genome. They are responsible for the repair of mistakes during normal DNA replication and for the repair of damaged DNA due to exposure to endogenous or exogenous mutagenes (Peltomäki and de la Chapelle, 1997; Gupta and Lutz, 1999; Peltomäki, 2001, Vogelstein and Kinzler, 2004). Caretaker genes are therefore involved in (1) the detection of DNA-damage and activating the repair machinery, (2) the repair of damaged DNA and (3) the inactivation of mutagenic molecules before they damage the DNA (Negrini et al., 2010; Ciccia and Elledge, 2010; Kastan, 2008; Friedberg et al., 2006). Mutation and inactivation of these genes involved in mismatch repair, nucleotide-excision repair, and base-excision repair, result in increased genomic instability and accumulation of additional mutations affecting oncogenes and TSGs (Kinzler and Vogelstein, 1997; Cahill, 1999; Roth and Gellert, 2000; Vogelstein and Kinzler, 2004). Stability genes are also involved in the control of processes affecting large portions of chromosomes, such as mitotic recombination and chromosomal segregation (Vogelstein and Kinzler, 2004).

MicroRNA

MicroRNAs (miRNAs) are evolutionarily conserved, small (~22 nucleotides), non-coding, endogenous RNAs that play an important role in the regulation of a variety of biological processes, such as cell differentiation, proliferation, organ development, the maintenance of stem cell potency, and apoptosis (Reinhart et al., 2000; Ambros, 2004; Chan et al., 2005; Cheng et al., 2005). miRNAs can function as oncogenes or TSGs in tumorigenesis by post-transcriptionally regulating of the expression of target genes involved in oncogenesis. Aberrant miRNA expression has been shown to correlate with the development of various human malignancies (Lu et al., 2005; Esquela-Kerscher et al., 2006; Volinia et al., 2006). One of the first miRNAs to be identified was Let-7. Let-7 has a tumor suppressor function through negative regulation of certain oncogenes such as *HMGA2* and *RAS* (Johnson et al., 2005; Mayr et al., 2007). Other well-known tumor suppressive miRNAs are mir-15a, mir-16-1 and mir-34. miRNAs may function as oncogenes by targeting TSGs. Two prominent examples are mir-21, which deregulates the expression of *PDCD4* (programmed cell death 4) and *PTEN* (Frankel et al., 2008; Meng et al., 2007), and the mir-17-92 cluster that targets genes involved in multiple apoptotic pathways (He et al., 2005). Each miRNA has the ability to target a large number of genes (up to 500 genes for each miRNA family) and approximately 60% of all mRNAs have one or more conserved binding site for miRNAs (Lewis et al., 2003; Krek et al., 2005). miRNAs are promising new diagnostic biomarkers of human cancers and in the future, they may also constitute new targets for antitumor therapies. However, the complex nature of miRNA networks in normal and cancer cells is a challenge to overcome before it eventually might be possible to develop such therapies.

The Hallmarks of Cancer

The delicate balance between cell proliferation and cell death that governs normal tissue is disrupted by genetic alterations in cancer cells. Tumor development proceeds through a succession of genetic changes in a Darwinian manner, where each genetic alteration confers a selective growth advantage over adjacent normal cells. In their classic assay, published in 2000, Hanahan and Weinberg suggest that every cancer, even though their genotypes differ, acquires six functional capabilities during a multistep process that allows the cancer cells to survive, proliferate and disseminate (Hanahan and Weinberg, 2000). The authors state that these six core hallmarks constitute the rules that govern the transformation of normal cells into cancer cells. The order in which these capabilities are acquired during tumorigenesis may vary significantly depending on cancer type (Hanahan and Weinberg, 2000). In a next generation version of *Hallmarks of cancer* published in 2011, the two potential and emerging hallmarks, reprogramming of energy

metabolism and evading immune destruction, are added to the six core hallmarks (Hanahan and Weinberg, 2011). Underlying the recently proposed eight hallmarks are genomic instability and inflammation, two enabling characteristics crucial for the acquisition of these hallmarks (Hanahan and Weinberg, 2011). Below is a short description of the eight hallmarks.

1. ***Sustaining proliferative signaling***. Normal cells are in need of and in strict control of growth-promoting signals from the extracellular environment when entering into a proliferative state. Cancer cells develop an ability to deregulate these signals and to reduce their dependence of the surrounding normal microenvironment, thereby taking control of their own destinies. This may be achieved in a number of alternative ways, they may (i) acquire a capability to produce and provide themselves and the surrounding tissues with growth signals, (ii) elevate the levels of receptor proteins on the surface of the cancer cells and (iii) deregulate the intracellular signaling cascades that translate these signals into action. These characteristics are often achieved by gain of function alterations of oncogenes, such as RAS, RAF and MYC, and their signalling pathways.

2. ***Evading growth suppressors***. Tissue homeostasis and the regulation of cell proliferation in normal tissues are maintained by multiple antiproliferative signals, such as soluble growth inhibitors and immobilized inhibitors in the extracellular matrix. Cancer cells must circumvent these signals and disrupt these antigrowth signaling networks. Most antiproliferative signals are associated with the two prototypical tumor suppressors pRB and TP53. They both play central roles in two key complementary regulatory circuits in the cell that govern cell proliferation and apoptosis.

3. ***Evading apoptosis***. The expansion of tumor cells depends not only on the rate of cell proliferation but also on the rate of attrition. Programmed cell death, triggered by extracellular or intracellular signals, is the major source of this attrition. Tumor cells have evolved a variety of strategies to limit or circumvent apoptosis. By far the most common strategy is loss of function mutations in the pro-apoptotic regulator TP53. Genetic alterations of this tumor suppressor gene are seen in more than 50% of human cancers (Harris, 1996). In addition, in certain specific circumstances, cell death through necrosis has been proven to be a genetically controlled process rather than a random one (Galluzzi and Kroemer, 2008; Zong and Thompson, 2006).

4. ***Limitless replicative potential***. Most mammalian cells carry an autonomous program that limits their multiplication. This limitation ensures that, after a certain number of doublings, the cells stop growing due to two distinct "barriers", i.e. senescence (a viable but nonproliferative state) and crisis (involving cell-death). The capability of replicative immortality most frequently involves deregulation of telomerase, a DNA polymerase that

protects the chromosome ends. Cancer cells must overcome the process of telomere shortening for a tumor to evolve.

5. ***Sustained angiogenesis***. Both normal cells and cancer cells depend on the vasculature and its supply of oxygen and nutrients to survive and function properly. In normal tissues, the outgrowth of new blood vessels (angiogenesis) is a tightly regulated and transiently active process. Cancer cells must acquire an angiogenetic ability in order to grow in size. An altered balance of angiogenic inducers, such as vascular endothelial growth factor-A (VEGFA), and angiogenetic inhibitors, such as thrombospondin-1 (TSP-1), can induce this angiogeneic switch (Hanahan and Folkman, 1996).

6. ***Tissue invasion and metastasis***. Eventually, neoplastic cells in most cancers acquire an ability to invade adjacent tissues and form metastases. This multistep process, termed invasion-metastasis cascade, begins with local invasion, followed by the dissemination of cancer cells into nearby vessels and transit to distant tissues/organs, and finally ends with formation of micrometastatic lesions and the subsequent development of macroscopic metastases (Talmadage and Fidler, 2010; Fidler, 2003). Metastases are the cause of more than 90% of all human cancer deaths. Alterations in the cellular microenvironment play an important role in both invasive and metastatic growth and are caused by deregulation of several classes of proteins including extracellular proteases, cadherins, cell-cell adhesion molecules (CAMs) and integrins (Aplin et al., 1998). One well-known such example is the loss of E-cadherin, a key molecule in cell-to-cell adhesion, in carcinomas.

7. ***Reprogramming of energy metabolism***. Increased cell growth and proliferation in neoplasms is achieved, not only by deregulation of cell proliferation but also by changing the energy metabolism in order to supply the cells with fuel during cell division and growth. In many cancer cells, this is achieved by limiting their energy metabolism to glycolysis, both in the presence and absence of oxygen. The use of glycolysis can be an advantage during hypoxic conditions in many tumors, but an increased glycolysis also allows the reorganization of glycolytic intermediates into different biosynthetic pathways required for cell proliferation.

8. ***Evading immune destruction***. The fact that the immune system is involved in the resistance to tumor formation and progression is widely accepted. According to the theory of immune surveillance, tumors must in some way escape from the immune system or limit its destructive cellular effects. In line with this concept are recent studies showing that patients with colon and ovarian tumors with a high infiltration of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells have a better prognosis than patients without (Pagés et al., 2010; Nelson, 2008).

The acquisition of the above-mentioned hallmarks of cancer is made possible by two enabling characteristics, that is developing genomic instability and, inflammation. Genomic instability may simply be explained by the fact that certain mutant genotypes confer a selective growth advantage over adjacent cells, enabling their outgrowth and dominance in the surrounding tissue. The inflammatory response seen in varying degrees in most neoplastic lesions contributes to multiple hallmark capabilities by supplying bioactive molecules to the tumor microenvironment, as well as inductive signals that contribute to the activation of hallmark-facilitating programs.

Cancer cytogenetics

Ever since the original studies of cell division in malignant tumors in 1890 by David Hansemann the role of genetic aberrations in tumor development has been a matter of debate (Hansemann, 1890). The persisting and paradigmatic view of the pathogenesis of cancer was described a quarter of a century later by Theodor Boveri (Boveri, 1914) who suggested that tumors originate from a single cell through acquired genetic changes. The first discovery of a chromosomal aberration specific for a neoplastic condition was the t(9;22)(q34;q11) translocation, also known as the Philadelphia chromosome (Nowell and Hungerford, 1960; Rowley, 1973). The subsequent extensive research in the field of cancer cytogenetics has provided us with a vast knowledge of chromosomal abnormalities in human neoplasms. The majority of these abnormalities are documented in The Mitelman Database of Chromosome Aberrations of Cancer, which contains data from nearly 60,000 cases with abnormal karyotypes (Mitelman, 2007a). Despite the fact that the majority of human cancers are solid tumors they constitute less than a third of all cytogenetically analyzed cases (Mitleman et al., 2007b). The majority of cytogenetically analyzed tumors are hematological neoplasms i.e. leukemias and lymphomas. Notably, the number of reported chromosomal abnormalities in solid tumors has increased considerably in recent years.

From the extensive cytogenetic and molecular genetic research performed so far, there are three important conclusions that may be drawn; (1) most if not all neoplasms are characterized by recurrent and often specific chromosome rearrangements; (2) certain rearrangements such as translocations, insertions, and invertions result in oncogenic gene fusions; and (3) the chromosomal and/or molecular genetic aberrations have important clinical implications. Chromosomal changes can be divided into two major categories, that is primary and secondary abnormalities. Primary abnormalities are considered to be of pathogenetic importance and may be found as the sole anomalies. They may also be tumor-type specific and can therefore be useful as diagnostic biomarkers. In contrast, secondary abnormalities are mainly of importance for tumor progression and may

have prognostic significance. Both primary and secondary abnormalities are regarded as non-random events and are rarely, if ever, seen in normal cells (Lenguaer et al., 1998; Mitelman, 2000; Mitelman et al., 2007a; Weinberg, 2007).

There are two major types of cytogenetic changes in neoplasia, i.e. numerical and structural aberrations. The former are mainly due to gains and losses of whole chromosomes during mitosis and commonly result in aneuploidy. Gene amplification is a special type of numerical change seen in certain types of neoplasms, involving copy number gains of one or more genes in a certain chromosome region. Amplified genes may be cytogenetically visible as homogeneously staining regions (hsr) or double minute chromosomes (dmin). Structural chromosome rearrangements include for example translocations, deletions, insertions, and inversions and several of these are now known to result in gene fusions.

Among solid tumors, recurrent tumor-type specific chromosome rearrangements have been found in for example thyroid tumors, renal cell carcinomas, sarcomas, benign mesenchymal tumors, and salivary gland tumors. Examples of such rearrangements are t(X;1)(p11;q21) and inv(X)(p11.2;q12) in papillary renal cell carcinomas (Sidhar et al., 1996; Clark et al., 1997), inv(10)(q11;q21) and t(10;17)(q11;q23) in papillary thyroid carcinomas (Pierotti et al., 1992; Sozzi et al., 1994), t(2;3)(q13;p25) in follicular thyroid carcinomas (Kroll et al., 2000), t(11;19)(q14.21-p11) in mucoepidermoid carcinomas (Nordqvist et al., 1994a), t(6;9)(q22.24;p12-23) in adenoid cystic carcinomas (Nordqvist et al., 1994b) and t(3;8)(p21;q12) and t(9;12)(p23;q14-15) in pleomorphic adenomas (Mark et al., 1980; Stenman, 2005).

Fusion oncogenes

Recurrent translocations commonly result in tumor-type-specific fusion oncogenes (Rabbitts, 1994; Lengauer et al., 1998; Åman, 1999; Rowley, 2001; Scandura et al., 2002; Mitelman et al., 2004; Stenman, 2005). Fusion oncogenes are considered as important early genetic events in neoplasia and are often associated with distinct tumor phenotypes. Because of their ability to disrupt multiple pathways and regulatory functions in the cellular machinery, they are sometimes thought of as "shortcuts" to malignancy, indicating their importance in cancer progression and clinical outcome in human cancer (Åman, 2005a). Recent estimates have suggested that gene fusions account for 20% of cancer morbidity in humans (Mitelman et al., 2011). There are two major types of fusion oncogenes and they act by alternative mechanisms (Figure 2). In the first type, the breaks occur within the coding regions of one or both genes, resulting in the formation of a new chimeric gene encoding a true fusion protein. In the second type, the breakpoints occur within the non-coding regions of both genes, resulting

in the exchange of 5'-regulatory elements and overexpression of a normal protein. This mechanism is known as "promoter swapping" (Kas et al., 1997; Åman 1999). Fusion oncogenes act mainly as positive regulators of cell growth and may for example function as aberrant transcription factors, constitutively activated tyrosine kinases, and as modulators of signal transduction (Rabbitts, 1994; Åman, 1999; Stenman, 2005).

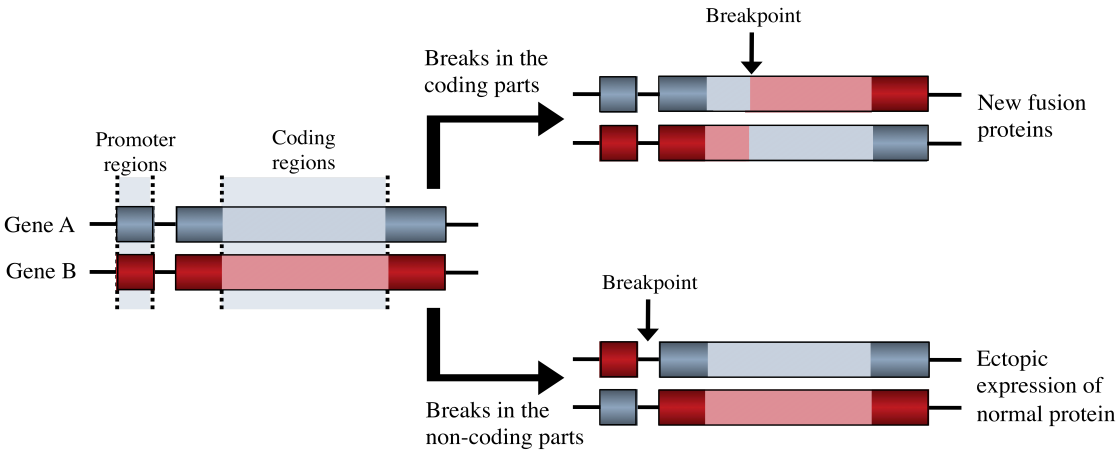

Figure 2. Generation of fusion oncogenes by two different mechanisms.

Fusion oncogenes in solid tumors

To date, the majority of fusion oncogenes have been identified in hematological disorders while only a limited number have been found in solid neoplasms. Recent studies, however, indicate that fusion oncogenes may be common also in solid tumors and that the low number of fusion oncogenes identified in solid tumors is due to an inability to discover these rearrangements rather than a true lack of gene fusions in carcinomas (Mitelman et al., 2004). It is therefore not surprising that several new gene fusions in solid tumors have been shown to result from intrachromosomal rearrangements and cryptic translocations/insertions that are not visible at cytogenetic level (Tomlins et al., 2005; Stenman et al., 2010). These observations strongly indicate that the number of gene fusions in solid tumors will increase in the near future. Recently, for example, recurrent fusions of the *TMPRSS2* gene to either of the oncogenic *ETS* transcription factor genes *ERG*, *ETV1* or *ETV4* were identified in more than 50 % of prostate cancers (Tomlins et al., 2005, 2006; Winnes et al., 2007a). One of the first fusion oncogenes identified in solid tumors was the *H4-RET* fusion in papillary thyroid carcinoma, generated by an inv(10)(q11q21) (Pierotti et al., 1992). Since then, the identification of fusion oncogenes in solid tumors has escalated (Mitelman et al., 2007). Oncogenic fusions may occur in both benign and malignant neoplasms and one such example is the recently identified *MYB-NFIB* fusion in adenoid cystic carcinomas of the breast and head and neck (*Paper III*) as well as in benign dermal cylindromas (Fehr et al., 2011). Another example is the *CRTC1-MAML2*

fusion, first identified in mucoepidermoid carcinomas of the salivary gland (Tonon et al., 2003; Enlund et al., 2004) and subsequently also in benign Warthin's tumors and clear cell hidradenomas of the skin (Enlund et al., 2004; Behboudi et al., 2006, Winnes et al., 2007b). Similarly, Möller and co-workers have identified an *EWSR1-POU5F1* gene fusion in both mucoepidermoid carcinomas and benign hidradenomas of the skin (Möller et al., 2008). Recently, an *EWSR1-ATF1* fusion gene was identified in hyalizing clear cell carcinomas of the salivary gland (Antonescu et al., 2011). This fusion was originally identified in clear cell sarcomas of tendons and aponeuroses with t(12;22) translocations (Zucman et al., 1993a). Another example of a fusion gene, which has been identified in several tumor entities, is the *ETV6–NTRK3* fusion, found in secretory breast carcinomas, mammary analog secretory carcinoma of the salivary gland, infantile fibrosarcoma, mesoblastic nephromate cells and acute myeloid leukemia (AML) (Knezevich et al., 1998; Rubin et al., 1998; Tognon et al., 2002; Skálová et al., 2010; Kralik et al., 2011). Taken together, the fact that the same fusion genes have been detected in benign and malignant tumors as well as in tumors of epithelial, mesenchymal and hematological origin indicates that certain fusion genes may not be as tumor specific as was previously believed.

In addition to the above-mentioned examples of fusion genes in benign tumors, recurrent rearrangements of 12q13-15 have been found in a variety of benign mesenchymal tumors, including uterine leiomyomas, lipomas, hamartomas of the breast and lung, fibroadenomas of the breast, angiomyxomas, endometrial polyps, and soft tissue chondromas as well as in pleomorphic salivary gland adenomas (Stenman, 2005 and references therein). These rearrangements target the transcription factor gene *HMGA2* and result in most cases in fusions where the 3′-end of HMGA2 is replaced by 3′-ends derived from a variety of other genes. Interestingly, pleomorphic adenomas are also characterized by fusion oncogenes involving another transcription factor gene, namely *PLAG1*. In all these fusions the result is deregulation of *PLAG1* expression due to promoter swapping with *CTNNB1*, *LIFR*, *TCEA1*, *CHCHD7* or *FGFR1* (Kaz et al., 1997; Voz et al., 1998; Åström et al., 1999; Asp et al., 2006; Persson et al., 2008).

Targeted therapies in cancer treatment

During the last decades, the efforts in cancer research have resulted in a remarkable progress in understanding the mechanisms behind the pathogenesis of cancer. As a result, we are now seeing the introduction of a rapidly growing number of targeted therapies. Two of the most prominent examples are Imatinib mesylate (Glivec®) and Trastuzumab (Herceptin®). Imatinib is a tyrosine kinase inhibitor used for treatment of patients with chronic myeloid leukemia (CML), as well as gastrointestinal stromal tumors (GISTs). It targets both the *BCR-ABL* fusion protein in CML and the tyrosine kinases encoded by mutated *KIT* and

PDGFRA in GIST (Buchdunger et al., 1996; Hirota et al., 1998; Buchdunger et al., 2000; Heinrich et al., 2003). Herceptin, on the other hand, is a monoclonal antibody that targets the ERBB2 growth factor receptor, which is amplified and activated in a subgroup of patients with breast cancer (Baselga et al., 1998). Both strategies represent cancer treatments in which the drug targets an activated "driver" oncogene. However, the clinical responses after targeted treatments are unfortunately often transitory, emphasizing the complexity of cancer. This complexity can at least in part be explained by the fact that the variety and number of genetic changes that cancer cells acquire involve several different and parallel signaling pathways. If a specific targeted therapeutic agent is not able to completely shut off a signaling pathway or only inhibits one of multiple parallel oncogenic pathways, the surviving cancer cells and their progeny may eventually adapt to the selective pressure of the therapy, thereby permitting the renewal of tumor growth and clinical relapse. Cancer cells may also, as a response to therapy, reduce their dependence on a given capability such as angiogenesis, and become more dependent on other capabilities that enabele them to resist the treatment (Ebos et al., 2009; Azam et al., 2010). To overcome this problem and to exhibit more efficient and durable therapies for human cancer, future targeted therapies will most likely depend on selective co-targeting of multiple signaling pathways activated in a specific tumor.

Clinical and cytogenetic features of Salivary Gland Neoplasms

Salivary gland tumors are a heterogenous group of neoplasms that constitute approximately 10% of the tumors in the head and neck region. According to the latest WHO classification (Barnes et al., 2005), salivary gland tumors comprise more than 30 different histological subtypes, ranging from benign tumors such as pleomorphic adenoma (PA) and Wartin tumor (WAT), to malignant tumors, such as adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC). The morphological diversity between different types of salivary gland tumors, as well as within a single tumor entity is often pronounced. Together with the fact that both benign and malignant salivary gland tumors can be characterized by highly specific and recurrent patterns of chromosome rearrangements, this makes salivary gland tumors very useful for studies of chromosome rearrangements, fusion oncogenes and tumor-type specificity (Stenman, 2005). The two most frequent malignant tumor types in the salivary glands are mucoepidermoid carcinoma and adenoid cystic carcinoma.

– ***Mucoepidermoid carcinoma***

MEC is the most common salivary gland malignancy and is characterized by epidermoid cells with columnar, clear cell or oncocytoid features, mucous cells, and intermediate cells in different proportions (Goode and El-Naggar, 2005). MEC most frequently occurs in middle-aged individuals (mean age approximately 45 years) and is slightly more common in women than in men (Auclair and Ellis, 1991; Goode and El-Naggar, 2005). The occurrence of MEC is slightly over-represented in the major glands of which 45% occur in the parotid glands, 7% in the submandibular glands, and 1% in the sublingual glands. Cytogenetic studies have shown that a high percentage of all MECs possess a t(11;19)(q21;p13) translocation, either as a sole abnormality or together with other structural and numerical abnormalities (Nordkvist et al., 1994a; Stenman, 2005). Molecular cloning of the t(11;19) translocation breakpoints resulted in the identification of the *CRTC1-MAML2* fusion oncogene (Tonon et al., 2003; Enlund et al., 2004). In the resulting fusion transcript, exon 1 of *CRTC1* is linked to exon 2-5 of *MAML2*. *CRTC1* (aka *MECT1*, *TORC1* and *WAMTP1*) encodes a cAMP response element binding protein (CREB) co-activator wheras *MAML2* (mastermind-like 2) encodes a nuclear protein that functions as a co-activator for *NOTCH* receptors (Tonon et al., 2003; Enlund et al., 2004). As a result of the t(11;19), a fusion is generated in which the N-terminal NOTCH-binding domain of MAML2 is replaced by the CREB-binding domain of CRTC1. The fusion protein is believed to activate or interact with target genes in both the cAMP/CREB- and NOTCH-pathway (Tonon et al., 2003; Enlund et al., 2003; Coxon et al., 2005; Wu et al., 2005; Komaiya et al., 2006). Expression of the fusion transcript is preferentially found in low-grade MECs as well as in a few cases of low-grade MECs that have progressed to high-grade MECs (Nagano et al., 2003). Several independent studies have shown that there is a clear association between the expression of the fusion transcript and tumor grade and that fusion-positive tumors are a less aggressive compared with fusion-negative tumors, suggesting that the CRTC1-MAML2 fusion is a reliable diagnostic and prognostic biomarker for MEC (*Paper I*; Miyabe et al., 2006; Anzick et al., 2009). Recurrent loss of the *CDKN2A* locus at 9p21 was recently also reported in fusion-positive MECs with a poor prognosis (Anzick et al., 2009).

– ***Adenoid cystic carcinoma***

ACC is one of the most common carcinomas of the salivary glands and comprises approximately 10% of all epithelial salivary gland neoplasms (El-Naggar et al., 2005). ACCs may also arise in other exocrine glands, such as the breast, cervix, vulva, and the tracheobronchial tree (Lin et al., 2002; Ellis et al., 2003). The tumors can be divided into three histological subtypes, tubular, cribriform, and solid, and most tumors display features of more than

one of these morphologic patterns (Hashimoto et al., 2002). Tumors with a predominantly cribriform and tubular growth pattern have a better prognosis than those with a more solid growth pattern (Batsakis et al., 1990; Fordice et al., 1999; Hashimoto et al., 2002; Bradley et al., 2004). Other clinicopathological parameters that have been proposed to be of importance in predicting the clinical behavior of ACC are lymph node involvement, advanced tumor stage, and perineural invasion (Fordice et al., 1999). Although ACC is a slow-growing cancer, its aggressive behavior usually results in a long-term poor prognosis. After 10-15 years, 80-90% of the patients are dead, due to this disease (El-Naggar et al., 2005 and references therein).

The molecular pathogenesis of ACC is still poorly understood and cytogenetic studies have indicated that ACC is characterized by a limited number of genetic alterations (Mitelman et al., 2011). We have recently shown that the recurrent translocation t(6;9)(q22-23;p23-24) in ACC consistently results in a *MYB-NFIB* gene fusion where the 5' part of the *MYB* oncogene is fused to the 3' part of the *NFIB* transcription factor gene (*Paper III*). The fusion is found in the majority of ACCs and is believed to be a primary event in the molecular pathogenesis of ACC. However, little is known about other genomic alterations of importance for the genesis and progression of ACC.

Clinical and cytogenetic features of Hidradenomas of the skin

Hidradenoma (HA) is a benign sweat gland tumor, often presenting as a solitary, slow-growing, solid or cystic intradermal nodule (Heenan et al., 1996; Brenn and KcKee, 2005). HAs may contain varying proportions of clear cells, and when these cells predominate the tumors are referred to as clear cell hidradenomas (CCH) (Wong et al., 1994). CCHs most frequently occur in middle-aged individuals and are usually located in the head and neck region or on the limbs. The tumors rarely recur or undergo malignant transformation. Cytogenetic studies of benign skin tumors are limited, but Gouronova et al. (1994) previously reported one case of CCH with a t(11;19)(q21;p13) translocation indistinguishable from the t(11;19) found in MEC. In this case, we were able to show that the translocation also resulted in and identical *CRTC1-MAML2* gene fusion (Behboudi et al., 2006). In a follow-up paper, we could also demonstrate that the *CRTC1-MAML2* fusion is recurrent in HAs and preferentially occurs in clear cell variants of this tumor type (*Paper II*).

OBJECTIVES OF THE THESIS

The general aim of this study was to characterize genetic alterations associated with subtypes of carcinomas and adenomas of the breast, salivary and sweat glands and to identify novel molecular targets for recurrent chromosome rearrangements. The specific aims were:

- to gain further insight into the molecular pathogenesis of the *CRTC1-MAML2* positive and negative mucoepidermoid carcinomas and to study the clinical significance of the *CRTC1-MAML2* fusion oncogene.
- to investigate whether the *CRTC1-MAML2* fusion oncogene is recurrent not only in MECs but also in cutaneous HAs.
- to identify the target genes of the recurrent t(6;9)(q22-23;p23-24) chromosomal translocation in ACC of the breast and head and neck and to study the molecular consequences of this rearrangement.
- to further characterize the genetic profile of ACC using high resolution arrayCGH analysis and identify potential target genes located in regions with recurrent copy number alterations.

MATERIALS AND METHODS

Tumor material

A total of 117 benign and malignant tumors were used in the studies included in this thesis. The samples include fresh frozen tumor tissues from 26 MECs, 40 ACCs, 3 WATs, and 25 non-ACC salivary gland carcinomas and invasive ductal carcinomas of the breast as well as paraffin-embedded material from 20 CCHs. All tumors were histopathologically re-examined to confirm the diagnosis. The tumors were classified according to the WHO-classifications of Head and Neck Tumours (Barnes et al., 2005), Tumors of the Breast and Female Genital Organs (Tavassoe'll and Devilee, 2003), and Tumours of the Skin (Weedon et al., 2005). Pertinent clinico-pathological information about the tumors is given in the respective papers. In addition, three MEC cell lines (Barsky et al., 1983), UT-MUC-1 (Grenman et al., 1992), UT-MUC-2, and NCI-H292 (ATCC no. CRL-1848; American Type Culture Collection, The Global Biosource CenterTM, Manassas, VA) were used in Paper I. HEK-293 cells (ATCC no. CRL-1573) and Cos-1 cells (ATCC no. CRL-1650) were used for transfection studies in Paper I, and the T-ALL cell line, MOLT-4 (ATCC No. CRL-1582), was used for miRNA transfection studies performed in Paper III.

Methods

The methods used in this thesis are well established and are described in the respective papers. They include cytogenic analysis, cell culture, fluorsescence in situ hybridization (FISH), spectral karyotyping (SKY), standard molecular biology techniques, RT-PCR, nucleotide sequencing, quantitative RT-PCR, immunohistochemistry, transfection of cultured cells, aCGH, and western blot. Hence, I will only make a few minor comments here. Paper IV in this thesis is based on array comparative genomic hybridization (aCGH) - a genome-wide scanning method originally developed for analysis at the chromosomal level but subsequently adopted to a microarray format using large genomic clones (BACs) or oligonucleotides as probes allowing detection of copy number alterations down to an intragenic level. The platforms used in Paper IV are high-resolution 244K (Human Genome CGH Microarray 244K; G4411B) and 1M (SurePrint G3 Human CGH Microarray 1M; G4447A) oligonucleotide arrays (Agilent Technologies Inc. Palo Alto. CA) with an average spatial resolution of 6.4 kb and 2.1 kb respectively. The possibility to detect genetic changes at such a level makes this method a very useful tool for detailed genomic profiling and identification of recurrent breakpoints, gains, and losses in tumors. However, aCGH also has certain limitations and it is important to note that when using this method it is not

possible to detect cases of copy-neutral loss of heterozygosity and that balanced rearrangements are impossible to detect unless they are associated with recurrent microdeletions or small duplications.

RESULTS AND DISCUSSION

Molecular classification of mucoepidermoid carcinomas-prognostic significance of the MECT1-MAML2 fusion oncogene (Paper I)

MECs of the salivary glands are characterized by a recurrent t(11;19)(q21;p13) translocation resulting in a *CRTC1-MAML2* gene fusion (Nordkvist et al., 1994; Tonon et al., 2003; Enlund et al., 2004). The *CRTC1-MAML2* fusion encodes a chimeric protein in which the Notch-binding domain of MAML2 is replaced by the CREB-binding domain of CRTC1 (a.k.a. MECT1, WAMTP1, and TORC1) through an exchange of N-terminals (Tonon et al., 2003; Enlund et al., 2004). In this study, a series of 29 MECs were cytogenetically and molecularly characterized to obtain further insight into the molecular and clinical consequences of the *CRTC1-MAML2* fusion in MEC. A t(11;19) translocation and/or a *CRTC1-MAML2* fusion was detected in more than 55% of the tumors. Detailed analyses using SKY and FISH revealed several cases with cryptic rearrangements resulting in gene fusions, indicating a higher frequency of fusion-positive tumors than suggested by conventional cytogenetic analysis. The second most common abnormality in this series was single or multiple trisomies, preferentially found in fusion-negative tumors.

To further characterize the CRTC1-MAML2 fusion protein we generated a polyclonal CRTC1-MAML2 antibody. Western blot analysis revealed expression of a CRTC1-MAML2 fusion protein of about 110 kDa in fusion-positive MEC cell lines. In contrast, this protein was not expressed in a fusion-negative cell line, demonstrating the specificity of the antibody. Immunostaining of fusion-positive primary MECs revealed predominant nuclear staining of all three major MEC-specific cell types, that is mucous cells, epidermoid cells, and intermediate cells. Taken together, these findings strongly indicate that the fusion is a basic event that occurs early in the transformation process.

Previous studies have shown that sustained expression of the *CRTC1-MAML2* fusion is essential for the growth of MEC tumor cells (Coxon et al., 2005; Komiya et al., 2006) and that the intracellular effects of the CRTC1-MAML2 fusion are complex, with deregulation of target genes in both the Notch and cAMP/CREB signaling pathways (Tonon et al., 2003; Enlund et al., 2004; Coxon et al., 2005; Wu et al., 2005). To obtain additional information about the molecular consequences of the fusion we analyzed the expression of three cAMP/CREB-responsive genes (*FLT1*, *NR4A2* and *CLDN7*) and two Notch-responsive genes (*HES1* and *HES5*) in primary MECs. We found clear differences in the expression pattern of four of these genes (*FLT1*, *NR4A2, HES1,* and *HES5*) in fusion-positive and fusion-negative tumors. An almost identical expression pattern was also found in WATs with and without the *CRTC1-MAML2* fusion. These findings are the first to demonstrate differential expression of cAMP/CREB and Notch target

genes in MECs *in vivo* and to provide evidence in support of a molecular classification of MECs based on the expression of the *CRTC1-MAML2* fusion.

Co-transfection studies of GFP-conjugated CRTC1-MAML2 and RedDS2-conjugated CREB1 in HEK-293 cells revealed co-localization in homogeneously distributed nuclear granules, indicating that activation of *CREB* might be an important mechanism through which CRTC1-MAML2 exerts its transforming activity. This is in line with previous studies showing that the fusion protein also co-localizes with p300/CBP (Wu et al., 2005) and that it can activate transcription of cAMP/CREB-regulated genes *in vitro* (Coxon et al., 2005; Wu et al., 2005).

MECs make up a heterogeneous group of tumors with a variable histology and clinical outcome. Different grading systems are used to distinguish high-grade MECs, with a poor prognosis, from low-grade MECs, with a more favorable prognosis (Auclair and Ellis, 1996; Aro et al., 2008). In an effort to overcome the problems and limitations of the existing grading systems, we investigated whether the *CRTC1-MAML2* fusion might serve as a more objective prognostic biomarker of MEC. Interestingly, we found that MECs in patients with fusion-positive tumors were preferentially highly differentiated low-grade tumors compared to MECs in fusion-negative patients. Fusion-positive patients had also a significantly lower risk of local recurrence, metastases and tumor-related deaths compared to patients with fusion-negative tumors (p=0.0012). When considering tumor-related deaths only, the estimated median survival for fusion-positive patients was more than 10 years compared to 1.6 years for patients with fusion-negative tumors. These initial observations, which were based on a limited number of tumors, have now been confirmed by several larger independent studies showing that the *CRTC1-MAML2* fusion preferentially occurs in low-grade MECs with a favorable clinical outcome (Okabe et al., 2006; Tirado et al, 2007; Miyabe et al., 2009; Seethala et al., 2010). The frequency with which this fusion occurs in MEC is still an unsettled question. Several independent studies indicate that the fusion is present in 34% to 81% in MEC (Okabe et al., 2006; Tirado et al., 2007; Miyabe et al., 2009; Seethala et al., 2011).

Recently, Fehr and co-workers showed that *CRTC3*, another member of the *CRTC* gene family, may also be fused to *MAML2* in MEC (Fehr et al., 2009). The composition of the *CRTC3-MAML2* fusion is very similar to that of the *CRTC1-MAML2* fusion, suggesting that the two fusions also have similar functions. In a subsequent study, the *CRTC3-MAML2* fusion was found in approximately 6% of MECs and the fusion was also associated with favorable clinicopathological features (Nakayama et al., 2009). These observations suggest that *CRTC3-MAML2* fusion-positive tumors represent a subgroup of *CRTC1-MAML2* negative MECs with a favorable prognosis (Nakayama et al., 2009).

Interestingly, Möller et al. recently also identifed a completely unrelated gene fusion in MEC (Möller et al., 2008). They found an *EWSR1-POU5F1* fusion is one out of three high-grade *CRTC1-MAML2* negative MECs as well as in several

cases of cutaneous hidradenoma (cf. *Paper II*). An identical fusion had previously been described in a single case of undifferentiated bone tumor, in line with the concept that *EWSR1*-containing gene fusions are known to be associated with sarcomas (Zucman et al., 1993b; Romeo and Dei Tos, 2010). Recent studies have also shown that hyalinizing clear cell carcinomas of salivary glands are characterized by an *EWSR1-ATF1* fusion (Antonescu et al., 2011), a rearrangement originally found in clear cell sarcomas of tendons and aponeuroses (Zucman et al., 1993a).

Taken together, the present and subsequent studies demonstrate that there are distinct differences in the clinical characteristics and outcome of MECs depending on the presence or absence of the *CRTC1-MAML2* fusion oncogene. These findings suggest that fusion-positive and fusion-negative MECs may in fact represent separate tumor entities and that *CRTC1-MAML2* is a novel biomarker that defines the MEC entity. The fact that low-grade MECs only rarely dedifferentiate into high-grade MECs (Nagano et al., 2003) suggests that the majority of fusion-negative MECs may be *de novo* high-grade tumors that constitute a heterogenous group of poorly differentiated carcinomas. In summary, this study demonstrates for the first time that a molecular classification of MECs based on the *CRTC1-MAML2* fusion is histopathologically and clinically relevant and that the fusion is a useful prognostic biomarker for this tumor type.

Frequent fusion of the CRTC1 and MAML2 genes in clear cell variants of cutaneous hidradenomas (Paper II)

Previous studies have shown that the *CRTC1-MAML2* fusion oncogene is a critical genetic alteration in MECs originating from salivary, bronchial, and thyroid glands (Tonon et al., 2003; Enlund et al., 2004; see above under *Paper I*). In addition to MEC, the *CRTC1-MAML2* fusion has also been found in two benign tumor entities, that is Warthins tumor (WAT) and cutaneous hidradenomas (HA) (Enlund et al., 2004; Behboudi et al. 2005; Winnes et al., 2006). The aim of this study was to investigate whether the previously reported hidradenoma with a t(11;19) translocation resulting in a *CRTC1-MAML2* fusion was a solitary case or whether the fusion may in fact also be a recurrent event in this tumor type. FISH-analysis of archival tumor material from 20 hidradenomas, using a dual-color break-apart *MAML2* probe, revealed rearrangements of *MAML2* consistent with a *CRTC1-MAML2* fusion in 10/20 (50%) hidradenomas. In the majority of fusion-positive tumors the fusion was detected in more than 90% of the tumor cells. Expression of *CRTC1-MAML2* transcripts was confirmed by RT-PCR in four of the 10 fusion-positive tumors. Immunostaining of the resulting CRTC1-MAML2 fusion protein revealed expression in the majority of tumor cells, including clear cells, poroid cells, and cells with dermoid and ductal differentiation. The results clearly demonstrate that the *CRTC1-MAML2* fusion

oncogene is recurrent also in cutaneous hidradenomas. As in MEC, the results also suggest that the fusion is a basic event that occurs early during tumorigenesis. The *CRTC1-MAML2* fusion appear to occur with similar frequency in both MEC and hidradenoma, and although they represent distinct tumor types, variants of these entities share certain morphological features such as epidermoid differentiation, clear cells and cyst formations. MECs may also occur in the skin and in a recent study, rearrangements of the *CRTC1* gene were also found in cutaneous MECs (Lennerz et al., 2009). Collectively, these observations thus reveal an important genetic link between hidradenoma and MEC and suggest common molecular pathways of importance for the development of both benign and malignant tumors of glandular origin.

It has been suggested that clear cell variants of hidradenoma are of apocrine derivation, in contrast to hidradenomas composed of poroid and cuticular tumor cells, which are suggested to be of eccrine derivation. To find out whether such a subclassification of hidradenomas could correlate with fusion gene status, we blindly reviewed all our cases. Interestingly, the *CRTC1-MAML2* fusion was only found in tumors containing various amounts of clear cells, whereas fusion-negative tumors were mainly composed of poroid and cuticular cells, indicating that the *CRTC1-MAML2* fusion is associated with the clear cell variant of hidradenoma. These findings raise the question of whether fusion-positive and fusion-negative hidradenomas may represent separate tumor entities. In the former cases, the *CRTC1-MAML2* fusion may be a useful diagnostic biomarker that can easily be detected by FISH in archival material.

In this context it should be noted that Möller and co-workers recently showed that a subset of cutaneous hidradenomas express an *EWSR1-POU5F1* gene fusion (Möller et al., 2008). The same fusion was also detected in a case of high-grade MEC. They suggested that the *EWSR1-POU5F1* fusion was preferentially associated with less well-differentiated tumors, which is in line with our observation of expression of *CRTC1-MAML2* in more highly differentiated MECs and hidradenomas. It will be interesting to find out whether *CRTC1-MAML2* and *EWSR1-POU5F1* affect the same or different cellular pathways.

Taken together, the present and previous results (*Paper I*, Okabe et el., 2006) raise the question of whether fusion-positive MECs, WATs and clear cell hidradenomas might originate from a common progenitor cell in salivary, bronchial and sweat glands, in line with their common derivation from the surface ectoderm (Larsen, 2001). The emerging data also indicate that the fusion can be etiologically correlated to histogenetically related, but morphologically different, benign and low-grade malignant tumor types.

Recurrent fusion of MYB and NFIB transcription factor genes in carcinomas of the breast and head and neck (Paper III)

We have previously identified a t(6;9)(q22-23;p23-24) translocation as a recurrent genetic alteration in ACC of the head and neck (Stenman et al., 1986; Nordkvist et al., 1994; Mitelman et al., 2011). In several cases, the t(6;9) translocation appears as the sole anomaly, indicating that it is a primary genetic alteration in this tumor type (Nordkvist et al., 1994). Our group has previously identified several gene fusions resulting from chromosome translocation in different types of benign and malignant salivary gland tumors (Stenman, 2005; Stenman et al., 2010). Since gene fusions appear to be relatively common in salivary gland neoplasms, we reasoned that also the t(6;9) translocation in ACC may generate such a fusion. We therefore performed cytogenetic and spectral karyotype analysis of 6 ACCs of the head and neck and identified a t(6;9) translocation in all cases. Using these tumors as the starting material, we performed a series of FISH experiments to further characterize the 9p23-24 and 6q24-25 breakpoints. FISH mapping of a series of yeast artificial chromosome (YAC) clones derived from 9p23-24 (Geurts et al., 1998) revealed that one YAC clone, which contains the *NFIB* gene as the only known gene, spanned the 9p breakpoint. Similarly, FISH analysis using a series of bacterial artificial chromosome (BAC) clones derived from 6q22-24 revealed that the 6q breakpoint was located within a 1.1 Mb region containing five genes, of which the *MYB* oncogene was the most obvious candidate gene. Based on the transcriptional orientations of these genes we reasoned that the t(6;9) translocation might generate a fusion consisting of the 5′-part of *MYB* linked to the 3′-part of the *NFIB* transcription factor gene. FISH experiments using the *NFIB*-containing YAC and a set of BACs containing the 5′-part of *MYB* revealed a fusion signal on the der(6) marker chromosome in an ACC with t(6;9) consistent with a *MYB-NFIB* gene fusion. The expression of *MYB-NFIB* fusion transcripts in this case was subsequently confirmed by RT-PCR. RNA from 10 additional ACCs, comprising 6 tumors derived from the head and neck and 4 from the breast, were screened for the fusion with RT-PCR to determine whether the fusion is recurrent in ACC. We were able to show that this was the case and that all 10 ACCs were fusion positive. In contrast, none of the 25 non-ACC tumor samples that were tested were fusion-positive. The predominant fusion transcript variants identified consisted of *MYB* exon 14 linked to *NFIB* exons 8c or 9. However, due to alternative splicing and different breakpoints we also identified 9 additional less common *MYB-NFIB* transcript variants.

To study the molecular consequences of the *MYB-NFIB* fusion on the expression of *MYB*, we performed quantitative RT-PCR on a series of fusion-positive ACCs. Using a TaqMan probe for *MYB* exons 1-2 we were able to demonstrate high overexpression of *MYB* in ACC relative to both normal salivary gland and 3 other types of salivary gland carcinoma. Analysis of the expression of *MYB* exons 14-15

revealed a significantly lower expression level, indicating that the increased expression of *MYB* in ACC is not due to overexpression of the wild-type allele. We could also confirm a high overexpression of MYB at protein level in 5 primary ACCs with *MYB* rearrangement.

The mechanism by which the *MYB-NFIB* fusion activates *MYB* is still incompletely understood. The minimal common region of *NFIB* fused to *MYB* is exon 9 which encodes the last 5 amino acids of the NFIB protein, and the minimal common region of *MYB* that is lost due to fusion is exon 15, which encodes the last 38 amino acids of the MYB protein. We expect that the contribution of the last 5 amino acids of NFIB, suggested to be critical for the correct function of the protein (Roulet et al., 1995), is likely to be limited. Nor do our data suggest that most fusions disrupt the C-terminal negative regulatory domain of MYB (encoded by exons 10-13). Instead we argue that deregulation of *MYB* might result from the loss of the 3′-part of the gene. The deleted 3′-UTR of *MYB* contains several binding sites for microRNAs miR-15a/16 and miR-150 that were recently shown to negatively regulate *MYB* expression (Xiao et al., 2007; Chung et al., 2008; Zhao et al., 2009). Interestingly, recent studies have shown that the *HMGA2* oncogene is activated by a similar mechanism, i.e. the loss of Let-7 miRNA binding sites in the 3′-UTR of *HMGA2* as a result of gene fusion (Lee et al., 2007; Mayr et al., 2007). In a small subset of these cases *HMGA2* is activated as a result of fusions with the same 3′-part of *NFIB* that is also fused to *MYB* (Geurts et al., 1998; Stenman, 2005).

To test this hypothesis, we first performed qPCR experiments demonstrating that miR-15a/16 and miR-150 are expressed in both ACC and normal salivary gland and breast tissues. Subsequently, we overexpressed miR-15a/16 and miR-150 in primary cultured fusion-positive ACC cells and in a T-cell acute lymphoblastic leukemia (T-ALL) cell line (MOLT-4) with *MYB* overexpression due to a duplication of *MYB*. Transfection of miR-15a/16 and miR-150 did not significantly affect *MYB* expression in primary ACC cells lacking these miRNA binding sites but resulted in a decreased expression of *MYB* in MOLT-4 cells with an intact *MYB* gene. This supports our hypothesis that the *MYB-NFIB* fusion may disrupt the repression of *MYB* through deletion of miRNA binding sites in the 3' UTR of the gene. However, we cannot exclude the possibility that loss of parts of the MYB regulatory domain or that sequences in the 3′-UTR of *NFIB* also may contribute to the deregulation of *MYB*. If proven correct, the fusion in ACC represents a second example of miRNA-directed repression of an oncogene activated by a chromosomal translocation.

Prior to our identification of the *MYB-NFIB* fusion there was little evidence supporting the notion that *MYB* actually is an oncogene rearranged in human cancer. Two recent studies showed that subsets of T-ALL are characterized by duplications of *MYB* or by t(6;7)(q23;q24) translocations placing an intact *MYB* gene under control of the *TCRB* regulatory sequences (Clappier et al., 2007; Lahortiga et al., 2007). Our group recently also showed that benign dermal

cylindromas, a tumor which shares certain morphological features with ACC, express *MYB-NFIB* gene fusions identical to those in ACC (Fehr et al., 2011). Together with previous observations of *CRTC1-MAML2* fusions in MEC and hidradenomas of the skin, these findings strengthen the evidence for common molecular pathways that are crucial for the development of both benign and malignant breast, salivary and adnexal tumors. Interestingly, a second gene fusion involving the *MYB* gene, that is *MYB-GATA1*, was recently identified in acute basophilic leukemia (ABL) and acute myeloid leukemia (AML) (Quelen et al., 2011; Belloni et al., 2011). In accordance with our study, the *MYB-GATA1* fusion results in overexpression of *MYB*. Taken together, these studies further emphasize the significance of *MYB* as an important human oncogene.

To study the molecular consequences of constitutive high-level expression of the *MYB-NFIB* fusion we performed qPCR analysis of 16 *MYB* target genes (Ramsey et al., 2008; Lang et al., 2005) in 7 *MYB-NFIB* fusion-positive ACCs. Fourteen genes involved in essential biological processes, such as apoptosis (*API5*, *BCL2*, *BIRC3*, *HSPA8*, *SET*), cell cycle control (*CCNB1*, *CDC2*, *MAD1L1*), cell growth/angiogenesis (*MYC*, *KIT*, *VEGFA*, *FGF2*, *CD53*), and cell adhesion (*CD34*) showed an unequivocal overexpression relative to normal salivary gland tissue. Using a bioinformatic approach, we were able to confirm overexpression of 4 of these genes, that is *API5*, *BCL2*, *CDC2,* and *MYC* and of *BCL2* and *CDC2*, respectively, in 2 independent ACC gene-expression data sets (Frierson et al., 2002; Patel et al., 2006). Furthermore, the majority of ACCs have previously been shown to overexpress *KIT* (El-Naggar et al., 2005).

In summary, our findings reveal that the *MYB-NFIB* fusion is a recurrent genetic event in ACC and that deregulation of *MYB* expression and its target genes are key oncogenic events in the pathogenesis of ACC. The *MYB-NFIB* fusion is expressed in ACCs derived from the breast, salivary glands, lacrimal glands, and ceruminal glands of the ear, indicating that the fusion is a true hallmark of this tumor type. Our findings also suggest new possibilities and opportunities for diagnosis and treatment of ACC and identify *MYB* as a candidate therapeutic target.

Combined genomic profiling and gene expression analysis reveal recurrent copy number alterations and candidate target genes in adenoid cystic carcinoma (Paper IV)

In Paper III we showed that the *MYB-NFIB* fusion oncogene is a characteristic and recurrent genetic alteration in ACC. However, little is known about other genetic alterations in ACC and previous analyses of CNAs in ACC using array- and chromosomal-based CGH are limited to a few smaller studies comprising about 130 cases, including both frozen and formalin-fixed tumor material (Freier et al., 2005; Vekony et al., 2007; Bernheim et al., 2008; Rao et al., 2008; Costa et a., 2010; Seethala et al., 2011). The purpose of this study was to extend and refine these initial studies by analyzing a series of 40 frozen head and neck ACC

samples with known *MYB-NFIB* fusion gene status, the largest cohort to date. Specifically, we wanted to identify recurrent CNAs and candidate target genes within these CNAs by integrating copy number and gene expression data. We also wanted to study the clinical significance of these alterations.

Using high-resolution oligonucleotide 244K and 1M arrays, we were able to identify novel recurrent CNAs and present significant refinements of several previously detected CNAs. A total of 274 partial genomic imbalances were identified, of which 35 were considered to be recurrent, that is found in >4 cases. Copy number losses were almost twice as common as gains (24 versus 11). In addition, 27 non-recurrent gains/losses of whole chromosomes were found, most of which occurred in grade III tumors. An extra chromosome 19 was the only recurrent whole chromosome gain found in five cases. Notably, gene amplification and homozygous deletion were only detected in one case each, suggesting that such aberrations are not likely to be of significant importance for the genesis and/or progression of ACC. In contrast, our findings indicate that the ACC genome is generally fairly stable and contains comparatively few CNAs. This is supported by the fact that 14 of the 24 grade I tumors had three or fewer CNAs per tumor. Even when analyzing a subset of the tumors (11 cases) using high-density 1 M arrays we were not able to detect any major recurrent changes that were not detectable with the lower-resolution (244K) arrays. Taken together, these observations indicate that, in addition to *MYB* activation, other alterations such as mutations and changes in the DNA methylation pattern may be of pathogenetic importance in ACC (Williams et al., 2006; Sasahira et al., 2011; Bell et al., 2011).

At least 35 recurrent CNAs were detected. The most frequently lost regions were 12q13.3, 6q24.1-q27, 12q13.11-q13.2, 12q13.3-q14.1, 9p22.3-p22.2, 11q23.3, 14q23.3-q24.3, 1p36.33-p35.3, 5q12.3-q13.2, and 5q13.3-q14.3, and the most frequently gained regions were 1q32.1, 9p24.3-p23, 22q11.21, and 22q13.1. The frequencies of these recurrent CNAs varied from 10-30%, which agrees with what has previously been reported in smaller series of ACCs (Freier et al., 2005; Bernheim et al., 2008; Rao et al., 2008). These numbers indicate that no CNAs are likely to be of pathogenetic importance for more than a subset of ACC patients. One explanation for the low frequency of genetic changes such as CNAs in ACCs is the fact that activation of *MYB* through gene fusion or other mechanisms is thought to be a major oncogenic event in the majority of ACCs (*Paper III*; Stenman et al., 2010). In line with this assumption, we found that 30 of 35 ACCs in this series were positive for the *MYB-NFIB* fusion by RT-PCR. Real-time quantitative PCR analysis of most of these cases demonstrated that *MYB* was highly overexpressed in the absolute majority of these cases (unpublished data).

The most prominent findings in the 40 ACCs were complex patterns of breakpoints and/or deletions/gains involving 6q, 9p, and 12q in particular. The rearrangements affecting 6q almost invariably resulted in losses of 6q24.1-q27,

which is in line with previous studies showing deletions of the terminal part of 6q in a subset of ACCs (Stenman et al., 1986; Sandros et al., 1988, 1990; Queimado et el., 1998; Rutherford et al., 2006). Loss of this region was considerably more common in grade II and III tumors compared with grade I tumors. Within this region, we identified three smaller potential MCRs of losses at 6q24.1-q25.2, 6q26, and 6q27 (defined by two tumors) that were lost in 30% of the cases. The 6q24.1-q27 region contains several putative tumor suppressor genes, including *PLAGL1*, *LATS1*, *PARK2, THBS2*, and *PDCD2* (Verrault et al., 1998; Streit et al., 1999; Xia et al., 2002; Kishi et al., 2002; Takahashi et al., 2005; Abdollahi, 2007; Baron et al., 2007; Veeriah et al., 2010). An integrated analysis of copy number losses in this region and the global gene expression pattern in four tumors revealed 13 genes, including *PLAGL1*, *MAP3K4,* and *RPSGKA2*, that were significantly down-regulated in tumors with 6q deletions compared to tumors without such deletions. Collectively, these analyses identified several potential target genes in 6q24.1-6q27 that merit further investigation. Genomic imbalances indicating breakpoints in *MYB* were identified in four cases; in three of these, the 3′-part of *MYB* was deleted, and in one case, there was a gain of one copy of *MYB*. All four cases with rearrangement/gain of *MYB* were positive for the *MYB-NFIB* gene fusion.

Genomic imbalances involving 9p were seen in 13 cases, including two MCRs of losses at 9p22.3-p22.2 and 9p24.2-p23 and one MCR with gain of 9p24.3-p23. The pattern of genetic imbalances involving 9p strongly indicates that *NFIB* is the target gene for these rearrangements and that the preservation of the 3′-end of *NFIB* is critical. Ten tumors had breakpoints in *NFIB* and in five of these, the segment 9pter-p23, including the 3′-part of *NFIB*, was gained. One particularly interesting case had a deletion of the entire 9p except for an approximately 600 kb segment including the 3′-end of *NFIB*. Five of the 10 cases were *MYB-NFIB* fusion positive and two were fusion negative (no data was available from the other cases).

The genomic imbalances affecting 12q, found in up to 30% of the cases, included loss of three MCRs located at 12q13.11-q13.2, 12q13.3, and 12q13.3-q14.1. The larger region spanning from 12q13.11-q13.2 includes the putative tumor suppressor genes *LIMA1* and *NR4A1* (Maul and Chang, 1999; Jiang et al., 2008; Ramirez-Herrick et al., 2011). Interestingly, global gene expression analysis of four ACCs with 12q deletions revealed *NR4A1* as one of 10 genes in 12q13.11-q13.2 that were down-regulated in tumors with loss of this region compared to those without 12q losses. Recent studies have shown that *NR4A1* is a potent tumor suppressor, involved in the coordination of proliferation, cell cycle arrest, apoptosis, and DNA-repair (Maxwell et al., 2006; Ramirez-Herrick et al., 2011). Collectively, these findings thus identify *NR4A1* as a potential target gene for the 12 deletions in ACC.

Another interesting observation in this study was the loss of an MCR at 1p36.33-p35.3 in a subset of tumors. This region is frequently deleted also in several other

tumor types, including neuroblastoma, breast cancer, melanoma, and colorectal cancer (Ragnarsson et al., 1999 and references therein). Notably, we only found 1p deletions in patients with grade II (one case) and grade III (four cases) tumors, which is in line with a previous chromosomal-based CGH study by Rao and co-workers (2008) showing that deletion of 1p32-p36 is a poor prognostic marker in ACC. Analysis of gene expression data from three tumors with loss of 1p36.33-p35.3 revealed down-regulation of 44 genes in this region compared to tumors without 1p deletions. Among these are the putative tumor suppressor genes *NBL1*, *CASP9* and *SFN* (Nakamura et al., 1997; Soengas et al., 1999; Mahwech, 2005). Several other candidate target genes, including *UBE4B*, *RUNX3*, and *PRDM2*, are also located in this region (Li et al., 2002; Krona et al., 2003; Geli et al., 2010). Interestingly, *SFN* (Uchida et al., 2004) and *RUNX3* (He et al., 2008; Sasahira et al., 2011) has previously been shown to be down-regulated in ACC. In the latter case, the down-regulation correlated with poor prognosis. This is in agreement with our observation that tumor-related deaths occurred in 3 of our 4 patients with 1p deletions for whom follow-up was available.

In summary, this study has revealed novel CNAs and significant refinements of previously described CNAs in ACC. We have also identified several new candidate target genes located within regions of recurrent CNAs. Further molecular analyses of these and other genes located in these regions may lead to identification of new genes of pathogenetic, prognostic, and therapeutic importance for ACC.

CONCLUSIONS

The main observations and conclusions of these investigations can be summarized as follows.

- A t(11;19)(q21;p13) translocation and/or a *CRTC1-MAML2* gene fusion is found in 55% of MECs. The fusion is preferentially expressed in low-grade tumors. The CRTC1-MAML2 fusion protein co-localizes with CREB in nuclear granules, suggesting that the fusion protein may act through activation of CREB. cAMP/CREB and Notch target genes are differentially expressed in fusion-positive and fusion-negative MECs.
- MECs may be molecularly classified based on the presence or absence of the *CRTC1-MAML2* fusion. Patients with fusion-positive tumors have a significantly lower risk of local recurrence, metastases, or tumor-related deaths compared to patients with fusion-negative tumors, indicating that *CRTC1-MAML2* is a useful biomarker in predicting the biological behavior of MEC.
- The *CRTC1-MAML2* gene fusion is recurrent in cutaneous hidradenomas and is associated with the clear cell variant of this tumor. The results indicate that *CRTC1-MAML2* is etiologically linked to benign and low-grade malignant tumors originating from diverse exocrine glands rather than being linked to a separate tumor entity.
- The recurrent t(6;9)(q22–23;p23–24) translocation in ACC consistently results in a *MYB-NFIB* gene fusion. The fusion is present in the majority of ACCs located in the breast and head and neck, indicating that it is a hallmark of this tumor type.
- The *MYB-NFIB* fusion results in loss of the 3′-end of *MYB*, including several conserved binding sites for miRNAs that regulate *MYB* expression negatively. The data indicate that deletion of these target sites may disrupt repression of *MYB* leading to overexpression of *MYB-NFIB* transcripts and protein and to activation of critical *MYB* target genes.
- The gain-of-function activity resulting from the *MYB-NFIB* fusion is a candidate therapeutic target.
- Copy number losses involving 12q, 6q, 9p, 11q, 14q, 1p, and 5q and copy number gains involving 1q, 9p, and 22q are recurrent in ACC. Several candidate target genes, including for example *NBL1*, *SFN*, *PLAGL1*, *PARK2* and *NR4A1*, are down-regulated in tumors with 1p, 6q or 12q deletions compared to tumors without such deletions. Losses of 1p36.33-p35.3 and 6q24.1-q27 are associated with high-grade tumors whereas losses of 14q23.2-q24.3 are exclusively seen in grade I tumors.

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