Studies of Fusion Oncogenes and Genomic Imbalances in Human Tumors

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To my mother and father

Doctrina superare morbos

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Abstract

Cancer is a genetic disease caused by mutations and chromosome rearrangements affecting oncogenes and tumor suppressor genes in particular. Molecular analyses of recurrent translocations in hematological disorders, as well as in certain solid tumor types, have shown that they frequently result in fusion oncogenes. These are key regulators of cellular transformation and play an important role in the initial steps of tumorigenesis. We have previously shown that recurrent translocations in pleomorphic salivary gland adenomas (PA) result in gene fusions involving the transcription factor genes *PLAG1* and *HMGA2*. Here we have used a combination of genomic techniques, including spectral karyotryping, FISH and high-resolution oligonucleotide array CGH, to (i) identify novel gene fusions in PA and carcinoma ex pleomorphic adenoma (Ca-ex-PA) and study their molecular consequences and the mechanisms by which they are generated, and (ii) characterize novel genomic imbalances in PA, Ca-ex-PA, and well-differentiated liposarcoma (WDLS) and identify genetic alterations associated with malignant transformation of benign PA.

Analyses of a series of 28 PA revealed novel *TCEA1-PLAG1* and *CHCHD7-PLAG1* gene fusions in one and three cases, respectively. The fusions were generated by cryptic, intrachromosomal 8q rearrangements in tumors with translocations or normal karyotype, leading to activation of *PLAG1* expression by promoter swapping/substitution. Our findings further emphasize the significance of *PLAG1* activation in PA and demonstrate that cryptic gene fusions are more common than previously anticipated.

We also studied a series of 16 PA with ring chromosomes of which 11 were shown to be derived from chromosome 8. Detailed analyses revealed that the latter consisted of amplification of a pericentromeric segment with recurrent breakpoints in *FGFR1* in 8p12 and in *PLAG1* in 8q12.1, resulting in novel *FGFR1-PLAG1* gene fusions. An alternative mechanism of *PLAG1* activation was found in two tumors with copy number gain of an intact *PLAG1* gene. These findings further illustrate the versatility of the *FGFR1* and *PLAG1* genes in tumorigenesis.

Analyses of 16 PA and Ca-ex-PA revealed amplification in dmin and hsr of a 30 kb minimal common sequence, encoding the three DNA-binding domains of *HMGA2* in 10 tumors. Co-amplification of *MDM2* was found in 9 tumors. Several tumors had amplification of cryptic *HMGA2-WIF1* gene fusions. *HMGA2* and *MDM2* were highly overexpressed in tumors with amplification. In general, PA showed significantly fewer genomic imbalances compared to Ca-ex-PA (3.8 vs. 24.5). The following alterations were suggested to be of importance for malignant transformation of benign PA: amplification of *HMGA2* and *MDM2*, deletions of 5q23.2-q31.2, gains of 8q12.1 (*PLAG1*) and 8q22.1-q24.1 (*MYC*), and amplification of *ERBB2*.

A novel WDLS-derived cell line with a giant marker chromosome showed amplification of the same 12q sequences as in PA and Ca-ex-PA, as well as sequences in 1q23.3-q44 and 13q32.1-q32.2. In the 12q amplicons, *MDM2* showed the highest level of amplification, followed by *LYZ* and *HMGA2* (5´-part). Several amplified genes, including *HMGA2* and *MDM2*, were highly overexpressed. The selective high-level amplification of the 5´-part of *HMGA2* suggests that this gene is also a major target of amplifications in WDLS.

Key words: cancer genetics, fusion oncogene, gene amplification, array CGH, PLAG1, HMGA2, FGFR1, MDM2, pleomorphic adenoma, carcinoma ex pleomorphic adenoma, liposarcoma

Original Papers

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

I. Asp J, Persson F, Kost-Alimova M, Stenman G. CHCHD7-PLAG1 and TCEA1-PLAG1 gene fusions resulting from cryptic, intrachromosomal 8q rearrangements in pleomorphic salivary gland adenomas.
Genes Chromosomes Cancer 2006;45:820-828.

II. Persson F, Winnes M, Wedell B, Andrén Y, Dahlenfors R, Asp J, Mark J, Enlund F, Stenman G. High-resolution array CGH analysis of salivary gland tumors reveals fusion and amplification of the *FGFR1* and *PLAG1* genes in ring chromosomes. *Submitted*

III. Persson F, Andrén Y, Winnes M, Wedell B, Nordkvist A, Dahlenfors R, Sjögren H, Mark J, Stenman G. Genome-wide high-resolution aCGH analysis of pleomorphic adenoma and carcinoma ex pleomorphic adenoma reveal genetic alterations associated with malignant transformation. *Manuscript*

IV. Persson F, Olofsson A, Sjögren H, Chebbo N, Nilsson B, Stenman G, Åman P. Characterization of the 12q amplicons by high-resolution, oligonucleotide array CGH and expression analyses of a novel liposarcoma cell line. *Submitted*

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Introduction

During the last quarter of a century we have witnessed a formidable evolution in the field of cellular and molecular cancer research. To date, a cascade of information, almost too vast for any human mind to comprehend, has been revealed. On the basis of molecular medicine and the rapid evolution of genetic techniques, researchers can now study thousands of genes and their products in a tumor. Identification of gene expression profiles with both diagnostic and prognostic potential is now possible. Hundreds of genes and proteins have been proven to participate in the origin of tumor disease. Cancer is not looked upon as merely one disease, but many. Consequently, there cannot be a single treatment for these different entities, but a combination of several different strategies is instead needed to defeat cancer. This is true today and will most probably also apply in the foreseeable future.

General aspects of cancer

In essence, cancer is a genetic disease (Boveri, 1914; Bishop, 1987; Weinberg, 1989; Hahn and Weinberg, 2002; Vogelstein and Kinzler, 2004). The normal cell is tightly regulated by homeostatic mechanisms that govern cell proliferation and growth. Cancer occurs as the result of a stepwise accumulation of genetic changes that liberate the neoplastic cell from normal growth control (Klein and Klein, 1985; Hahn and Weinberg, 2002). Genetic changes commonly found in cancer cells are for instance gene mutations and chromosomal aberrations, such as deletions and translocations. Changes in the genome of somatic cells over a prolonged period of time may ultimately result in the transformation of these cells into tumor cells (Figure 1.). Cancer arises because these genomic changes provide the cells with a growth and survival advantage in comparison to the surrounding cells (Vogelstein and Kinzler, 1993; Yokota, 2000; Hahn and Weinberg, 2002).

The number of mutations required to convert a normal cell into a cancer cell appear to vary between different cell and tumor types (Hahn et al., 1999, Hanahan and Weinberg, 2000). Colorectal cancers, for instance, are caused by at least five to six genetic changes (Vogelstein and Kinzler, 1993).

Approximately 10% of all cancers are hereditary, i.e. the person carries a genetic predisposition to develop one or more tumors. This predisposition consists of a germ line mutation that is present in every cell in the body. The remaining 90% of cancers are sporadic and occur when a somatic cell has accumulated a sufficient number of mutations for a tumor to develop.

Most, if not all, tumors or neoplasms are thought to be of monoclonal origin; that is, they all originate from a common ancestral cell (Knudson, 1985; Wainscoat and Fey, 1990). When the cancer cell acquires its unlimited proliferative potential, its progeny may accumulate additional mutations over time. This may lead to genetic instability, selection, and clonal expansion "of the fittest", resulting in a heterogeneous tumor cell population (Nowell, 1976; Woodruff, 1983; Heppner, 1984).

Tumors can be classified as benign and malignant. Benign tumors are often surrounded by a capsule and grow in a non-invasive manner, respecting the boundaries of the surrounding tissues. In contrast, malignant tumors lack capsules and are capable of invading surrounding tissues and forming distant metastases. It is widely accepted that malignant tumors have acquired additional genetic changes compared with benign tumors (Kinzler and Vogelstein, 1996; Yakota, 2000; Mitelman et al., 2007a) (Figure 1.).

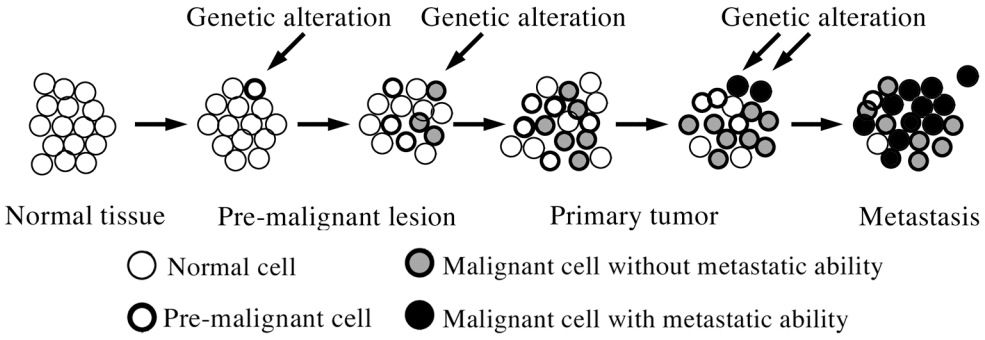

Figure 1. Genetic alterations accumulate in a stepwise manner over a prolonged period of time during tumorigenesis and tumor progression. *Modified from (Yokota, 2000).*

Neoplasias can be further classified as solid tumors and hematopoietic malignancies. The latter include leukemias and lymphomas. Solid tumors may be derived from epithelial, mesenchymal or neuroectodermal cells. Benign solid tumors are named using the suffix –oma, e.g. an adenoma originates from glandular structures and a lipoma from lipomatous tissue. Malignant epithelial tumors are referred to as carcinomas and malignant mesenchymal tumors are referred to as sarcomas. The most common human malignancies are carcinomas, which are responsible for more than 80% of cancer-related deaths in the Western world (Weinberg, 2007).

"The hallmarks of cancer"

A contemporary classical essay with the above title was published in 2000 by Hanahan and Weinberg (Hanahan and Weinberg, 2000). In this paradigm-creating work, the authors state the rules that govern the transformation of normal human cells into cancer cells. In normal tissues, there is a delicate balance between cell proliferation and cell death, or apoptosis. The genetic alterations acquired by cancer cells disrupt this pivotal balance. The ultimate result is a growth advantage for the tumor cells, and clonal expansion in a Darwinian manner at the expense of their normal counterparts. Although many neoplasms have different genotypes, they may all be manifestations of the six acquired capabilities which, taken together, dictate the creation of malignant cells.

1. *Self-sufficiency in growth signals*. Normal cells are unequivocally dependent on growth signals for their ability to proliferate. Cancer cells are not dependent on these molecules, because they can generate them themselves. This is made possible in different ways, such as through the secretion of mitogenic growth factors that act in a autocrine manner, and through the deregulation of growth factor surface receptors, extracellular matrix receptors, and intracellular signaling pathways.

2. *Insensitivity to antigrowth signals*. Tissue homeostasis and regulation of cell proliferation are maintained in normal tissues by antigrowth signals, such as soluble growth inhibitors and cell-matrix and cell-cell interacting molecules. In most cells, the retinoblastoma protein, pRB1, is a major factor controling cell cycle progression from G1 to S phase (Weinberg, 1995). This pathway is disrupted in many cancers, thus rendering the tumor cells insensitive to antigrowth signals that normally function along the pRB1 pathway.

3. *Evading apoptosis*. Programmed cell death or apoptosis is a delicately regulated pathway for making unwanted cells disappear. The program may be triggered by DNA damage, signaling imbalance, survival factor insufficiency and hypoxia (Evan and Littlewood, 1998). Cancer cells can evade apoptosis in a variety of ways. The most common mutated pro-apoptotic regulator is the *TP53* tumor suppressor gene. Functional inactivation of the TP53 protein is seen in more than 50% of human cancers, resulting in disruption of sensoring of DNA damage, oncogene overexpression and hypoxia that normally trigger the apoptotic machinery (Harris, 1996; Levine, 1997).

4. *Limitless replicative potential*. Most mammalian cells carry an autonomous program that limits their multiplication. Regardless of the signaling pathways described above, this proliferative limiting program must also be disrupted before a tumor can evolve. Mammalian cells in culture have a limited potential for replication (reviewed in Hayflick, 1997). After a certain number of doublings, the cells stop growing – a phenomenon termed senescence. If the cells are forced to multiply further, by disabling TP53 or pRB1, for example, they may enter a second state termed crisis, which is characterized by massive cell death and end-to-end fusion of chromosomes. Occasionally, single immortalized cells with limitless replicative potential may develop (Wright et al, 1989). The number of cell divisions for a normal cell is limited by the shortening of its telomeres. The erosion of telomeres leads to unprotected chromosomal ends resulting in the crisis and eventual cell death (Counter et al, 1992). Cancer cells evade this process in different ways, for instance, by upregulating the telomerase activity (Bryan and Cech, 1999).

5. *Sustained angiogenesis*. All cells are dependent on the vasculature for nutrients and oxygen. Cancer cells are no exception to this. Angiogenesis – the growth of new blood vessels – is tightly regulated in normal tissues. In order to progress to a larger tumor, cancer cells must acquire an angiogenic ability

(Hanahan and Folkman, 1996; Bouck et al., 1996). It has been suggested that this takes place through an "angiogenic switch" during which tumors, probably through altered gene expression, acquire the ability to alter the balance of pro-angiogenic factors, such as VEGF and FGFs, and of anti-angiogenic factors, such as thrombospondin-1 (Hanahan and Folkman, 1996).

6. *Tissue invasion and metastasis*. During the development of most types of cancer in man, the tumor cells acquire the ability to invade the surrounding tissues and form metastases. Metastases are responsible for 90% of all cancer-related deaths (Sporn, 1996). Cancer cells forming metastases are also dependent on the five other acquired capabilities. The genetic and biochemical alterations leading to the formation of metastases are largely unknown. Both invasive and metastatic growth involve alterations in the cellular microenvironment. Several classes of proteins that mediate these alterations are deregulated in cancer cells; they include extracellular proteases, cadherins, cell-cell adhesion molecules (CAMs), and integrins (Aplin et al., 1998). E-cadherin is a major suppressor of invasion through the transmission of antigrowth signals via cell-cell interactions. In many epithelial tumors, E-cadherin function may be lost by the inactivation of the E-cadherin gene or by the proteolysis of the extracellular cadherin domain. The loss of function of E-cadherin is probably a major step in the acquisition of invasive capabilities in many epithelial cancers (Christofori and Semb, 1999). Recently, the transcription factor *TWIST1*, a master regulator of embryonic morphogenesis, was identified as a major breast cancer metastasis gene (Yang et al., 2004).

The above mentioned acquired capabilities and characteristics of cancer cells are shared by most, if not all, human cancers. The order in which these alterations occur during tumorigenesis may vary significantly from one tumor to another (Hanahan and Weinberg, 2000). The genes associated with the above-mentioned capabilities can be divided into three major classes; i.e oncogenes, tumor suppressor genes, and DNA repair genes (Bishop, 1991; Vogelstein and Kinzler, 2004).

Oncogenes

Proto-oncogenes are a heterogeneous group of genes encoding proteins involved in the positive regulation of cell growth and proliferation. Oncogenes represent aberrant or mutated forms of proto-oncogenes. They are dominant genes, i.e. mutation of one allele is normally sufficient to cause disrupted or deregulated expression (gain of function mutation). The activation and aberrant expression of oncogenes contribute to abnormal cell proliferation and tumorigenesis (Bishop, 1991; Vogelstein and Kinzler, 2004). The first oncogene was identified more than thirty years ago by Stehelin and co-workers (Stehelin et al., 1976). They discovered that the transforming potential of the Rous sarcoma virus in avian species was due to a normal cellular gene named *src.* Today, hundreds of oncogenes have been discovered.

Proteins encoded by oncogenes act in many different ways and at different levels in the cellular machinery. They can function as growth factors (e.g. *PDGFB*), growth factor receptors (e.g. *ERBB*), signal transduction molecules (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*) (Doolittle et al., 1983; Waterfield et al., 1983; Wells and Bishop, 1988; Brown and Cooper, 1996; Barbacid, 1987; Bos, 1989; reviewed in Bishop, 1991; reviewed in Tashiro et al., 2007).
Oncogenes can be activated through several different mechanisms, including point mutation, gene amplification, chromosomal rearrangements, such as translocations, insertions and inversions, and viral transduction (Bishop, 1991; Weinberg, 1994). Examples of oncogenes activated by point mutations include members of the *RAS* gene family, that is *HRAS, KRAS* and *NRAS*. Overexpression of RAS promotes cell cycle progression and leads to the activation of different signaling pathways (Barbacid, 1987; Stacey and Kung, 1984; Macaluso et al., 2002). Interestingly, mutated *RAS* oncogenes have been detected at high frequencies in many human tumor types, including pancreatic carcinoma, lung cancer, colon cancer, and thyroid carcinoma (Bos, 1989; Macaluso et al., 2002).
ERBB2, EGFR and *MYCN* are examples of oncogenes that may be activated by gene amplification. *EGFR* is amplified in malignant gliomas (Humphrey et al., 1988) and *MYCN* in neuroblastoma (Schwab et al., 1983). *ERBB2* encodes a growth factor receptor with tyrosine-kinase activity that is amplified and overexpressed in subsets of breast cancer, ovarian cancer and malignant salivary gland tumors (Slamon et al., 1989, Stenman et al., 1991, Paper III). In recent years, new cancer treatment strategies, including monoclonal antibodies against surface receptors, such as ERBB2, have evolved. Breast cancer patients with amplification and overexpression of *ERBB2* can thus benefit from trastuzumab (Herceptin®) treatment (Baselga et al., 1998).
There are numerous neoplasms, both hematological disorders and solid tumors, in which oncogenes are activated by chromosomal rearrangements, i.e. translocations, inversions and insertions. The most well-known example is activation of the *ABL* oncogene in chronic myeloid leukemia (CML), where a recurrent t(9;22)(q34;q12) translocation (which generates the *Philadelphia chromosome*) (Nowell and Hungerford, 1960; Rowley, 1973) results in the fusion of the 5´part of *BCR* to, in most cases, exons 2 to 11 of *ABL*. The chimeric fusion protein, BCR-ABL, has a constitutively activated tyrosine kinase and is found in virtually all patients with CML. The fusion protein induces cellular effects such as for example reduced apoptosis and self-sufficiency in mitogenic signaling (Chissoe et al., 1995; Melo, 1996; Fernandez-Luna, 2000). Interestingly, the treatment of CML patients with Imatinib mesylate (Glivec®), a tyrosine kinase inhibitor targeting the BCR-ABL fusion protein (Buchdunger et al., 1996), has produced remarkable results during the past few years. This represents a new anti-cancer treatment strategy in which a drug specifically targets the driving force, the activated oncogene, in a neoplasia. Indeed, Imatinib inhibits not only BCR-ABL in CML but also the constitutively activated tyrosine kinases encoded by *KIT* and *PDGFRA* in gastrointestinal stromal tumors, GISTs. Also in this tumor type, inhibition of the tyrosine kinase activity produces dramatic treatment effects (Buchdunger et al., 2000; Hirota et al., 1998; Heinrich et al., 2003).

Tumor suppressor genes (TSG)

TSG (or gatekeeper genes) function as transcriptional regulators, inhibitors of proliferation, repressors of cell cycle progression, inducers of apoptosis, and maintainers of genomic stability. Loss or functional inactivation of these genes and their products contribute to deregulated proliferative control in tumor cells (Levine, 1997; Macleod, 2000; Kinzler and Vogelstein, 1997; Vogelstein and Kinzler, 2004; Sherr, 2004).

Mutations or deletions of TSG lead to loss of function (loss of function mutations). Since tumor suppressor genes are recessive genes at the cellular level, both the maternal and the paternal alleles must be inactivated. This inactivation may be achieved in three ways, i.e. by deletion, mutation or DNA methylation (Jones and Laird, 1999; Weinberg, 2007). Both hereditary and sporadic tumors may have inactivated TSG. In hereditary tumors, a germline mutation is inherited in one allele of a TSG, as demonstrated in Knudson's "two-hit" model (Knudson, 1971). The other allele is subsequently lost or inactivated in a somatic cell (Sherr, 2004). As a result, inheritance of a single mutant allele dramatically increases tumor susceptibility, since only one additional mutation/deletion is required for complete loss of function (Sherr, 2004).

Examples of well-known TSGs are *TP53, RB1*, *APC*, *NF1*, and *NF2*. Perhaps the most commonly mutated gene in human cancer is *TP53* (Hollstein et al., 1994; Olivier et al., 2002). The TP53 protein mediates cell cycle arrest and is necessary for induction of apoptosis in response to DNA damage (Levine, 1993, 1997; Haffner and Oren, 1995). Inactivation of TP53 leads to genomic instability due to accumulation of mutations (Weinberg, 2007). Because of its key functions in the cellular response to DNA damage, it has been named "guardian of the genome" (Lane, 1992). Indeed, mutations in *TP53* are found in more than 50% of human cancers (Sherr, 2004).

The first TSG to be characterized was *RB1*, the retinoblastoma gene (Friend et al., 1986). The gene product, pRB1, is a nuclear phosphoprotein that represses genes regulating programs governing cell cycle progression, apoptosis and differentiation, for example. It exerts its major effect(s) by physically interacting with transcription factors, such as the E2Fs, during the G1 phase of the cell division cycle. Phosphorylation of pRB1 by cyclin-dependent kinases (CDKs) cancels RB1-mediated repression of the target genes (Sherr, 2004; Weinberg, 2007).

The second most commonly altered genomic region after *TP53* is perhaps the *CDKN2A/2B* locus at 9p21 (Ruas and Peters, 1998). The *CDKN2A* locus encodes two tumor suppressor proteins generated by different reading frames; $p16^{INK4A}$, which inhibits the phosphorylation of pRB1 by cyclin D-dependent kinases (CDK4 and 6), and $p14^{ARF}$, which binds to the *MDM2* protein and stabilizes TP53 (Quelle, 1995; Sharpless and DePinho, 1999; Sherr, 2000, 2001, 2004). Collectively, pRB1, TP53, $p16^{INK4A}$, and $p14^{ARF}$ form part of a network that monitors mitogenic signaling and restrains growth-promoting signals (Sherr, 2004). Inactivation of this signaling network appears to occur in most forms of human cancer (Nobori et al., 1994; Sherr, 2000, 2004; Hahn and Weinberg, 2002).

DNA repair genes

The main function of DNA repair genes, also referred to as caretaker or stability genes, is to ensure and maintain the integrity of the genome. Mutation and inactivation of these genes results in the accumulation of mutations and increased genomic instability (Kinzler and Vogelstein, 1997, Vogelstein and Kinzler, 2004; Roth and Gellert, 2000; Cahill et al., 1999). Depending on the cause of the DNA damage, different repair mechanisms are activated in the cell. Caretaker genes are involved in mismatch repair (e.g. *MSH2* and *MLH1* mutated in hereditary nonpolyposis colorectal cancer, HNPCC), nucleotide-excision repair and base-excision repair. They are responsible for repairing subtle mistakes made by the DNA replication machinery or induced by exposure to endogenous or exogenous mutagens (Peltomäki and de la Chapelle, 1997; Peltomäki, 2001; Vogelstein and Kinzler, 2004; Gupta and Lutz, 1999). Other caretaker genes control processes responsible for mitotic recombination and chromosomal segregation (Kinzler and Vogelstein, 2004).
Since caretaker genes keep genetic alterations to a minimum, inactivation of these genes increases the likelihood of other genetic alterations to occur, including activation of oncogenes and inactivation of TSGs (Vogelstein and Kinzler, 2004; Friedberg, 2003; Cahill, 1999).

Chromosome aberrations in human neoplasia – cancer cytogenetics

Chromosome abnormalities in neoplastic cells have been studied for well over a century (Hansemann, 1890). The first chromosome aberration that was found to be specific for a particular neoplastic condition, was the t(9;22)(q34;q11) identified in CML (Nowell and Hungerford, 1960; Rowley, 1973). In recent decades, extensive research in cancer cytogenetics has yielded a vast knowledge of chromosomal abnormalities in human neoplasms. The Mitelman Database of Chromosome Aberrations in Cancer contains more than 52,000 karyotypically abnormal cases (Mitelman et al., 2007a). The majority of cytogenetically analyzed cases are hematological neoplasms, i.e. leukemias and lymphomas, and less than one third are solid tumors (Mitelman et al., 2007b). For example, in acute myeloid leukemia, the number of reported abnormal karyotypes in 2004 was close to 11,000, including 126 recurrent balanced aberrations, 86 fusion genes, and 97 rearranged genes. This number was close to the total number of reported karyotypically abnormal solid tumors (Mitelman et al., 2004). In this context, it should also be noted that hematological disorders only constitute a few percent of all neoplasms in man.
It is a well-established fact that a high frequency of neoplastic disorders are characterized by clonal, acquired chromosome abnormalities, in particular chromosome translocations and deletions. There are different types of cytogenetically detectable gross chromosomal rearrangements in neoplasia (Rabbitts, 1994; Lengauer et al., 1998):

- Numerical aberrations: gains and losses of whole chromosomes during mitosis result in aneuploidy. Numerical abnormalities are found in nearly all major human tumor types (Mitelman et al., 2007a).

- Structural aberrations: physical rearrangements of genetic material through breakage and incorrect rejoining of chromosomes. Examples of structural abnormalities are translocations, inversions, insertions and deletions. Translocations can be balanced (reciprocal), with no loss of genetic material, or unbalanced (non-reciprocal), with loss of parts or whole chromosome arms.

- Gene amplification: may be seen at the cytogenetic level as homogeneously staining regions (hsr), double minute chromosomes (dmin) and sometimes as ring or giant marker chromosomes. Gene amplification results from preferential replication of segments of chromosomal DNA which can vary in size and number of copies (Weinberg 2007; Papers II-IV).

All the above mentioned alterations commonly occur in specific tumor types and are rarely, if never, seen in normal cells (Lengauer et al., 1998; Weinberg, 2007; Mitelman, 2000; Mitelman et al., 2007a).
Chromosome changes can be divided into two major categories; primary and secondary abnormalities. Primary abnormalities are considered to be of pathogenetic importance. They are often tumor specific and may be found as the sole anomalies. Since primary chromosome abnormalities may be tumor specific, they may be useful as diagnostic markers. In contrast, secondary chromosome abnormalities are thought to be more closely related to tumor progression rather than tumor development. Indeed, in leukemias, secondary changes have been shown to be of prognostic importance, e.g. an extra *Philadelphia chromosome* and trisomy 8 in CML (Griesshammer et al., 1997). Both primary and secondary changes are regarded as non-random abnormalities. A third category of chromosome changes can be referred to as cytogenetic noise. These are random changes that occur during tumor progression as a result of genomic instability.
It is now a well-established fact that most, if not all, neoplasms studied in sufficient numbers show recurrent chromosome aberrations. (Mitelman, 2004; Mitelman et al., 2005).
In hematological disorders, a classic example of this kind of aberration is the t(8;14)(q24;q32) in Burkitt's lymphoma (Dalla-Favera, 1982; Taub et al., 1982). Examples of recurrent structural abnormalities in epithelial tumors 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)(q11q21) and t(10;17)(q11;q23) in papillary thyroid carcinomas (Pierotti et al., 1992; Sozzi et al., 1994), t(11;19)(q14-21;p11) in mucoepidermoid carcinomas (Nordkvist et al., 1994a), t(6;9)(q22.24;p13-23) in adenoid cystic carcinomas (Nordkvist 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). Mesenchymal tumors of soft tissue and bone are cytogenetically similar to hematological disorders in many respects. They both often have rather simple karyotypes and frequently show tumor type specific chromosome changes. For instance, Ewing's sarcomas frequently show a recurrent t(11;22)(q24;q12) translocation (Delattre et al., 1992; reviewed in Lazar et al., 2006), myxoid/round cell liposarcoma a t(12;16)(q13;p11) (Crozat et al., 1993) and dermatofibrosarcoma protuberans a t(17;22)(q22;q13) (Simon et al., 1997). Benign mesenchymal tumors

such as for example lipomas and uterine leiomyomas frequently show translocations with recurrent breakpoints in 12q14-15, e.g. t(3;12)(q27;q14) in lipomas and t(12;14)(q14;q23) in uterine leiomyomas (Petit et al., 1996; Schoenmakers et al., 1999).
Molecularly, these balanced chromosome translocations often result in fusion oncogenes. Intense research during the last few decades has revealed that fusion oncogenes, are indeed key regulators of cellular transformation (Rabbitts, 1994; Rowley, 2001).

Fusion oncogenes

Previous studies of chromosome translocations in particular, in both hematological malignancies and solid tumors, have revealed that they commonly result in tumor-specific fusion oncogenes (Rabbitts, 1994; Rowley 2001; Lengauer et al., 1998; Åman, 1999; Scandura et al., 2002; Mitelman et al., 2004; Stenman, 2005). Two major types of fusion genes have been identified. In the first type, breakpoints within the coding regions of one or both genes result in the formation of a chimeric gene encoding a true fusion protein. In the second type, the breakpoints occur in the non-coding regions of both genes, resulting in the exchange of 5´-regulatory control elements and the upregulation of a normal, but ectopically expressed protein. This latter mechanism is referred to as promoter swapping or promoter substitution (Kas et al., 1997; Åman, 1999), and often results in high expression of a normally silent or strictly regulated 3´partner gene and the subsequent downregulation of the 5´partner gene which is normally expressed (Kas et al., 1997).
Available data suggest that chromosome translocations and the subsequent formation of fusion oncogenes represent early and pivotal genetic changes in tumor development (Åman, 1999; Rowley, 2001; Scandura et al., 2002; Mitelman et al., 2007b). Fusion oncogenes may act as "shortcuts" to malignancy, where a single mutational event may disrupt multiple pathways and regulatory functions in the cellular machinery (Åman, 2005a). Importantly, additional genetic changes are still necessary for tumorigenesis to occur (Åman, 2005b; Mitelman et al., 2007b). It should also be pointed out that fusion genes may be more common than anticipated from gross chromosomal rearrangements and "what meets the eye", since several gene fusions have turned out to be cryptic (Mitelman et al., 2007b; Tomlins et al., 2005; Paper I).
The majority of fusion oncogenes have so far been identified in hematological disorders while only a limited number have been found in solid tumors. Recent studies have, however, indicated that gene fusions may also be common in solid tumors, particularly in epithelial tumors. As an explanation, it has been proposed that the relationship between hematological and solid neoplasms is biased as a result of methodological problems, and that there may be a linear correlation between the number of fusion genes and the number of reported cases with an abnormal karyotype (Mitelman et al., 2004).
Fusion genes occur in both benign and malignant neoplasms. In addition to the *BCR-ABL* fusion in CML, the *MYC* gene fusions in Burkitt's lymphoma are among the most well-known fusions in myelo- and lymphoproliferative disorders. These fusions result from translocations in which the coding sequences of the *MYC* gene at 8q24 are

juxtaposed to the constitutively active immunoglobulin (*IG*) heavy- or light-chain loci (e.g. *IGH* at 14q32) (Dalla-Favera, 1982; Taub et al., 1982; Rowley, 2001). As a result, the *MYC* gene becomes constitutively activated (ar-Rushdi et al., 1983). Activation of *MYC* is a key event in the transformation of B-cells to Burkitt's lymphoma (Adams et al., 1985, Rowley, 2001).

In solid tumors, one of the first fusion oncogenes described was the *H4-RET* fusion created by an inv(10)(q11q21) in papillary thyroid carcinomas (Pierotti et al., 1992). Another example of a gene fusion in a carcinoma is the *CRTC1-MAML2* fusion in mucoepidermoid carcinomas of the salivary glands (Tonon et al., 2003; Enlund et al., 2004). Interestingly, recent studies have shown that *CRTC1-MAML2* positive mucoepidermoid carcinomas have a much more favourable prognosis compared with fusion-negative tumors (Behboudi et al., 2006; Okabe et al., 2006). Moreover, the same gene fusion has also been identified in benign epithelial tumors, including Warthin's tumor (a.k.a. cystadenolymphoma) and clear cell hidradenoma of the skin (Enlund et al., 2004; Behboudi et al., 2005; Winnes et al., 2007a).

Recently, recurrent fusions of the prostate-specific gene *TMPRSS2* to the oncogenic *ETS* transcription factor genes *ERG*, *ETV1* and *ETV4* were discovered in prostate cancer (Tomlins et al., 2005, 2006). These cryptic fusions are found in more than 50% of prostate cancers and are probably the most common gene fusions found in human cancer (Tomlins et al., 2005, 2006; Winnes et al., 2007b). Interestingly, *ETS* gene fusions are also found in Ewing's sarcoma, in which *EWSR1* is fused to different members of the *ETS* family, including *ERG* and *ETV1* (Sorensen et al., 1994; Jeon et al., 1995).

Fusion genes have also been identified in benign tumors, of both mesenchymal and epithelial origin (Åman, 1999; Stenman 2005; Young and Narita, 2007). For example, subsets of lipomas, uterine leiomyomas, pulmonary chondroid hamartomas and pleomorphic adenomas, are characterized by recurrent rearrangements of 12q13-15 in which the transcription factor gene *HMGA2* is the target. Multiple rearrangements, mainly translocations, have been reported (reviewed in Åman, 1999; Stenman, 2005; Young and Narita, 2007). Interestingly, in lipomas, pulmonary chondroid hamartomas, and soft tissue chondromas, identical gene fusions have been found, namely *HMGA2-LPP* resulting from the t(3;12)(q27-28;q14-15) (Petit et al., 1996; Rogalla et al., 2000; Dahlén et al., 2003). Further, *HMGA2-NFIB* fusions have been found in pleomorphic adenomas and in lipomas (Stenman, 2005; Nilsson et al., 2005).

Fusion oncogenes exert their major effects as positive regulators of cell growth. In both hematological disorders and solid tumors, they may function as aberrant transcription factors, constitutively activated tyrosine kinases, and as modulators of signal transduction (Rabbitts, 1994; Åman, 1999; Stenman, 2005).

DNA amplification

DNA amplification is defined as a selective increase in copy number of DNA sequences. This results in an increase in gene dosage (hence the term gene amplification) and is an important mechanism for upregulation of gene expression. In this way, the cell can synthesize specific gene products in amounts that far exceed the transcriptional capacity of the normal diploid genome (Schwab, 1999). Gene

amplification is an essential mechanism of oncogene activation and drug resistance (Alitalo and Schwab, 1986; Knuutila, 1998; reviewed in Schwab, 1998; Stark et al., 1989). The sizes of the amplified sequences (amplicons) may vary from a few hundred kilobases to several megabases (Stark et al., 1989). At the cytogenetic level, gene amplification may be visible as:

- Double minute chromosomes (dmin) are 1-2 Mb long acentric stretches of circular DNA (Coquelle et al., 1998). Due to lack of centromeric function, they show an irregular mitotic segregation and a resulting uneven distribution to daughter cells. This often results in an extensive variation in number of dmin between tumor cells.

- Homogeneously staining regions (hsr) are linearly amplified sequences either found at the normal cytogenetic position of the sequence or inserted into other chromosome locations (Schwab, 1999). Less intercellular variation is seen here, because the amplified DNA is integrated into one or more chromosomes. Amplified sequences can exist as both dmin and hsr within a given cell population, but they are rarely seen together in the same cells.

- Ring chromosomes and giant marker chromosomes, which are abnormal, often C-band-negative chromosomes that may contain large, complex amplicons (Schwab, 1999, Gisselsson, 2000a).

Our knowledge of the mechanisms of gene amplification is still limited. What we observe in human tumor cells is merely the result of gene amplification rather than the mechanisms creating it. According to one theory, amplification may be initialized by double-strand breaks at fragile sites or by telomere dysfunction followed by breakage-fusion-bridge (BFB) cycles (Gisselsson et al., 2000b; Hellman et al., 2002, Albertson, 2006). Under the appropriate selection (perhaps losses generated by the BFB cycle), this may generate intrachromosomal amplification (Hellman et al., 2002, Schwab, 1999; Albertson; 2006). Other theories suggest that alternative mechanisms to BFB cycles are responsible for gene amplification in tumors (Kuwahara et al., 2004). For example, a replication-excision model for *MYCN* amplification has been proposed, explaining the findings of hsr and dmin in neuroblastoma. DNA containing *MYCN* will undergo a local round of extra replication, followed by excision and the formation of extrachromosomal dmin. Subsequently, the dmin may be integrated into a chromosome and amplified in situ (Savelyeva and Schwab, 2001). Oncogenes activated by amplification have been found in many types of neoplasia, both in hematological disorders and in solid tumors. One of the most well-known examples is *MYCN* amplification in neuroblastoma (Schwab et al., 1983). *MYCN* amplification has been shown to predict a poor patient outcome (Seeger et al., 1985; Rubie et al., 1997) and therapeutic approaches are in part based on the *MYCN* status. *MYCN* amplification is also found more infrequently in small cell lung cancer, retinoblastoma and malignant gliomas, for example (reviewed in Schwab, 1998).
Another example of oncogene amplification in human cancer is *ERBB2* amplification in breast cancer (Guan et al., 1994). A smaller fraction of primary breast cancers show

amplification of this gene. *ERBB2* is also a significant predictor of overall survival and time to relapse (Slamon et al. 1987, 1989; Al-Kuraya et al., 2004). Today, patients with breast cancer are analyzed with FISH for the detection of *ERBB2* gene amplification. If the gene is amplified, the patients may be treated with the monoclonal antibody trastuzumab (Herceptin®), which specifically targets the ERBB2 tyrosine kinase receptor (Baselga et al., 1998; Mass et al., 2005).
In certain types of sarcomas, and less frequently also in neuroblastomas and gliomas, amplified sequences derived from 12q13-15 are observed (Oliner et al., 1992; Dal Cin et al., 1993; Pedeutour et al., 1993; Corvi et al., 1995; Reifenberger et al, 1993). Amplified genes in this region include *GLI*, *DDIT3*, *SAS/TSPAN31*, *CDK4*, *HMGA2*, and *MDM2* (Forus et al., 1994; Berner et al., 1996; Dei Tos et al., 2000; Pedeutour et al., 1994, 1999; Gisselsson et al., 1998). Interestingly, MDM2 can bind and functionally inactivate TP53 (Oliner et al., 1993), a key player in the cellular response to DNA damage. This pathway is often defective in tumor cells with gene amplification (Livingstone et al., 1992; Yin et al., 1992; Chernova et al., 1995).
In summary, gene amplification is likely to be initiated by DNA double-strand breaks (preferably at fragile sites) that are not correctly repaired. This may occur in cells lacking normal cell cycle checkpoints as well as in cells with dysfunctional telomeres, malfunctioning replicative machinery, and/or a defective response to DNA damage (Stark, 1993; Smith et al., 1995; Schwab, 1999; Hellman et al., 2002; Albertson, 2006). It is important to remember that gene amplification by itself is not sufficient; the genes in question must also be expressed in order to contribute to tumorigenesis.

Clinicopathologic and cytogenetic aspects of pleomorphic adenoma and carcinoma ex pleomorphic adenoma

Salivary gland tumors are a heterogeneous group of epithelial neoplasms including more than 35 histological subtypes, according to the most recent WHO classification (Barnes et al., 2005). The spectrum of tumors ranges from completely benign tumors, like pleomorphic adenoma (PA), to high-grade carcinomas like adenoid cystic carcinoma (ACC), adenocarcinoma, epidermoid carcinoma, and carcinoma ex pleomorphic adenoma (Ca-ex-PA). Salivary gland tumors show a pronounced morphological complexity and diversity, sometimes also with overlapping histologic patterns between different tumor entities (Seifert, 1991; Stenman, 2005). Moreover, certain benign tumors such as pleomorphic adenomas may occasionally also progress to malignancy. These features make the above tumor types useful for studies of chromosome rearrangements, fusion oncogenes and tumor-type specificity.
The pleomorphic adenoma (PA) is the most common type of salivary gland tumor (Eveson et al., 2005a), accounting for about 60% of all salivary gland neoplasms (Eveson et al., 2005b). About 80% of PA occur in the parotid gland, 10% in the submandibular gland, and 10% in the minor salivary glands of the oral cavity and the upper respiratory and alimentary tracts. PA is a benign, slow-growing tumor showing a remarkable degree of morphological diversity, including epithelial and myoepithelial cells forming a variety of patterns in an often mucoid/myxoid,

chondroid or hyalinized matrix. Although it is a benign tumor, it can cause problems in clinical management due to its tendency to recur and the risk of malignant transformation (Ca-ex-PA). PAs are likely to originate from a pluripotent, intercalated duct cell (Stenman, 2005). PAs are treated by surgical excision and, if completely removed, the prognosis is excellent.

Extensive cytogenetic and molecular genetic studies have been performed on PA. More than five hundred tumors have been cytogenetically analyzed and have revealed a highly specific and recurrent pattern of chromosome abnormalities, mostly translocations (Mark et al., 1980; Sandros et al., 1990; Bullerdiek et al., 1993; Mark et al., 1997). Cytogenetically, four major subgroups of PA have been identified (Stenman, 2005): (1) tumors with rearrangements of 8q12 (39%), the most common aberration being the t(3;8)(p21;q12) translocation found in almost half of the cases. This translocation was the first tumor specific translocation identified in a benign human tumor (Mark et al., 1980). The remaining cases in this subgroup show a variety of structural rearrangements, mainly translocations, involving a large number of translocation partners, some of which are recurrent; (2) the smallest subgroup (8%) comprises tumors with rearrangements of 12q14-15. Here, the most common aberration is a t(9;12)(p12-22;q13-15) or an ins(9;12)(p12-22;q13-15) (Stenman et al., 1994), but there are also variant translocations with several translocation partners; (3) tumors with non-recurrent clonal changes (23%). Finally, the fourth subgroup (4) comprises tumors with an apparently normal karyotype (30%). Among these chromosomal aberrations, the t(3;8) and t(9;12) translocations and variants thereof are regarded as tumor-specific, non-random abnormalities in PA (Stenman, 2005).

Ca-ex-PA is defined as a PA from which an epithelial malignancy is derived (Gnepp et al., 2005). Ca-ex-PA constitute about 6% of all pleomorphic adenomas and 12% of all malignant salivary gland tumors (Gnepp et al., 1993). Malignant transformation is more likely to occur in long-standing, large tumors. The malignant component of Ca-ex-PA is often a poorly differentiated or undifferentiated adenocarcinoma, but virtually any type of carcinoma may be found (Lewis et al., 2001; Gnepp et al., 2005). Invasive Ca-ex-PA are usually highly malignant tumors; up to 50% of patients develop recurrences and up to 70% local or distant metastases (Gnepp et al., 2005). Our knowledge of the genetic events leading to malignant transformation of benign PA to Ca-ex-PA is scarce (Röijer et al., 2002; DiPalma et al., 2005, Righi et al., 1994, Fowler et al., 2006) and there are no reliable molecular markers that can predict the risk of malignant transformation.

Target genes in PA with recurrent chromosome changes

Previous studies have shown that the target gene in PA with t(3;8)(p21;q12) is the transcription factor gene *PLAG1* in 8q12 (Kas et al., 1997). The translocation results in promoter swapping between *PLAG1* and *CTNNB1* (in 3p21). The breakpoints invariably occur in the 5´noncoding sequences of both genes, leading to activation of *PLAG1* expression and reduced expression of *CTNNB1*. *PLAG1* is developmentally regulated with expression restricted to certain fetal tissues (lung, liver and kidney), while ß-catenin (encoded by *CTNNB1*) is a ubiquitously expressed protein functioning as an interface in adherence junctions, as well as in the WG/WNT

signaling pathway (Peifer, 1997; Willert, 1998). The PLAG1 protein contains seven canonical C_2H_2 zinc fingers at the N-terminus and a serine-rich C-terminus that exhibits transactivation capacity (Kas et al., 1997, 1998).

In addition to the *CTNNB1-PLAG1* fusion, four other fusions have been identified in PA: *LIFR-PLAG1* resulting from a recurrent t(5;8)(p13;q12), *TCEA1-PLAG1* and *CHCHD7-PLAG1* resulting from intrachromosomal cryptic rearrangements in 8q12, and *FGFR1-PLAG1* resulting from ringchromosome formation (Voz et al., 1998; Åström et al., 1999; Papers I and II). The fusions result in overexpression of a normal PLAG1 protein due to promoter swapping.

Interestingly, *PLAG1* has also been shown to be the target gene in lipoblastomas with 8q12 rearrangements (Åström et al., 2000; Hibbard et al., 2000). Lipoblastomas are benign fat cell tumors occurring mainly in children (Enzinger and Weiss, 1995). So far, two fusion variants have been identified: *HAS2-PLAG1* and *COL1A2-PLAG1* resulting from an intrachromosomal rearrangement joining band 8q12 with 8q24, and a t(7;8), respectively.

The second major target gene in PA is *HMGA2*, identified in tumors with 12q14-15 rearrangements (Geurts et al., 1997a, 1998). *HMGA2* belongs to the high mobility group (HMG) gene family, encoding heterogeneous proteins that are non-histone components of chromatin (Grosschedl et al., 1994). The expression of *HMGA2* is usually very low in adult tissues such as lung, kidney, CD34-positive stem cells, and normal salivary gland tissue. The expression is essentially restricted to embryonic tissues (Zhou et al., 1995; Rommel et al., 1997; Gattas et al., 1999). The HMGA2 protein contains three DNA-binding domains, a spacer domain and a highly acidic C-terminal domain. It functions as an architectural transcription factor, regulating transcription by binding to the minor groove of AT-rich DNA (Reeves and Nissen, 1990; Wolffe, 1994). Two major fusion partner genes of *HMGA2* have been identified in PA, *FHIT* and *NFIB* (Geurts et al., 1997a, 1998). The *HMGA2-FHIT* fusion gene results from a complex 3;10;12-translocation fusing exons 1-3 of *HMGA2* to exons 9 and 10 of the *FHIT* gene in 3p14.2; thirty-one C-terminal amino acids of the FHIT protein replace 26 amino acids of the acidic tail of HMGA2. *FHIT* (fragile histidine triad gene) is a member of the superfamily HIT of necleotide binding proteins. It has previously been shown to be fused to *TRC8* in hereditary renal cell carcinoma (Gemmill et al., 1998). The most common gene fusion in PA with 12q14-15 abnormalities is *HMGA2-NFIB* resulting from a t(9;12) or an ins(9;12) (Geurts et al, 1998). The consequence of this fusion is that the C-terminus of HMGA2 is replaced by the last five amino acids of the NFIB protein. NFI proteins are involved in transcriptional regulation of a variety of viral and cellular genes (Roulet et al., 1995).

Interestingly, *HMGA2* has also been identified as the target gene in a variety of benign mesenchymal tumors with 12q14-15 abnormalities, i.e. lipomas, uterine leiomyomas, hamartomas of the breast and lung, fibroadenomas of the breast, angiomyxomas, endometrial polyps and soft tissue chondromas (Ashar et al., 1995; Schoenmakers et al., 1995, 1999; Kazmierczak et al., 1995; Petit et al., 1996, 1999; Kurose et al., 2000; Mine et al., 2001; Broberg et al., 2002; Dahlen et al., 2003). At least eight fusion partners of *HMGA2* have been identified in mesenchymal tumors; *LPP*, *LHFP*, *RDC1*, *HEI10*, *ALDH2*, *COX6C*, *RAD51B*, and *EBF* (Stenman, 2005; Nilsson et al., 2006). *HMGA2* has also been implicated in malignancies such as liposarcomas,

osteosarcomas, acute lymphoblastic leukemia and lung carcinomas (Berner et al., 1997; Xu et al., 2004; Sarhadi et al., 2006).
In cases with breakpoints within *HMGA2* or in the 3´ non-coding region, the separation of the three DNA-binding domains from the acidic domain and the AUUUA motifs in the 3´-UTR leads to activation of the gene. The AUUUA repeats are potential mRNA-destabilizing motifs, the loss of which may lead to stabilization of *HMGA2* transcripts (Geurts et al., 1997a, Geurts et al., 1997b; Borrmann et al., 2001; Decker and Parker, 1995). Breakpoints may also occur outside the gene and it has been suggested that this leads to disruption of 5´- or 3´- regulatory sequences, resulting in ectopic expression of a normal HMGA2 protein (Geurts et al., 1997a; Schoenberg Fejzo et al., 1996; Röijer et al., 1999; Stenman, 2005).

Clinicopathologic and genetic aspects of well differentiated liposarcoma
Well-differentiated liposarcoma (WDLS) is the most common subtype of liposarcoma (Dei Tos et al., 2000; Dei Tos and Pedeutour, 2002; Pedeutour and Foa, 2002). WDLS or atypical lipomatous tumors (ALT) are locally aggressive tumors that do not metastasize unless they undergo dedifferentiation. The most important prognostic factor for ALT/WDLS is anatomic location; tumors located in deep anatomic sites such as the retroperitoneum, spermatic cord and mediastinum have the highest recurrence rates. WDLS occurring in the retroperitoneum often show a mixed morphological pattern and may for example contain myxoid areas, chronic inflammation, or a prominent fibrous component. This may occasionally cause diagnostic problems.
The cytogenetic hallmark of ALT/WDLS is supernumerary ring and/or giant rod marker chromosomes found either as the sole anomalies or in combination with other more or less complex aberrations (Pedeutour et al., 1994, 1999; Gisselsson et al., 1998; Mitelman et al., 2007a). The rings and giant markers consistently contain amplified sequences derived from chromosome 12q14-15. Several other chromosomal regions, such as 12q21-22 and 1q21-25, are often co-amplified together with 12q14-15 (Pedeutour et al., 1999; Kresse et al., 2005). Genes commonly amplified and implicated in the pathogenesis of ALT/WDLS include *MDM2*, *HMGA2*, *CDK4*, and *SAS*/*TSPAN31* (Dei Tos et al., 2000; Pedeutour et al., 1994, 1999; Gisselsson et al., 1998).

Genome-wide scanning techniques – an evolution from chromosomes to basepairs
The evolution of genome-wide scanning methods has been truly remarkable during the past few years. Chromosome banding has been the standard cytogenetic technique since the early 1970s (Caspersson et al., 1970). Although rather crude, this was the first "whole-genome" scanning method developed. The resolution of cytogenetics is at best 5-10 Mb (Shaffer and Bejjani, 2004). To increase the resolution, molecular cytogenetic methods such as fluorescence in situ hybridization (FISH), have been developed. With these methods it became possible to identify cryptic rearrangements and to characterize breakpoints in structural chromosome rearrangements in detail, even down to the gene level (Cremer et al., 1988; Gray and

Pinkel, 1992). In the late 1990s, the FISH technique was further developed with multicolor FISH and spectral karyotyping (SKY). Using whole-chromosome painting probes in 24-color format, numerical and structural chromosome aberrations involving all human chromosomes may be detected at a few megabases of resolution (Schröck et al., 1996, 1997; Speicher et al., 1996). A disadvantage of SKY is, however, that small deletions, insertions and intrachromsomal duplications can not be detected (Shaffer and Bejjani, 2004).

The next milestone in the development of genome-wide scanning technologies was the introduction of comparative genomic hybridization (CGH) (Kallioniemi et al., 1992). This technique was originally developed for analysis at the chromosomal level, but it has recently been adopted to microarray format (aCGH) using large genomic clones (BACs) or oligonucleotides as probes (Pinkel et al., 1998; Snijders et al., 2001; Brennan et al., 2004). Using this technique, losses and gains/amplifications of genomic sequences can be detected down to gene level. aCGH may also, as demonstrated in Papers II and III, be very useful for chromosome breakpoint mapping and the identification of novel fusion oncogenes.

Aims of the study

The aims of this thesis can be summarized as follows:

- to identify novel gene fusions in PA and Ca-ex-PA and study the mechanisms by which such fusions are generated

- to determine the derivation and genetic content of ring chromosomes in PA

- to identify genes amplified in PA and Ca-ex-PA with cytogenetic signs of gene amplification and to identify recurrent genetic alterations associated with malignant transformation of benign PA

- to molecularly characterize the 12q amplicon in a novel WDLS-derived cell line and to study the impact of gene amplification on the expression of potential target genes

Material and Methods

A total of 66 benign and malignant human tumors were used for the cytogenetic, molecular cytogenetic and molecular genetic studies in this thesis, including 55 PA, 10 Ca-ex-PA and one WDLS. The karyotypes of all cytogenetically analyzed tumors are shown in Papers I-IV. All tumors were classified according to the WHO-classifications of Head and Neck Tumors (Barnes et al., 2005) and of Soft Tissue and Bone Tumors (Fletcher et al., 2002). Histopathological re-examination of the tumors confirmed the original diagnoses of all cases. The clinico-pathological characteristics of the tumors are presented in Papers I-IV. The cytogenetic, molecular cytogenetic, and molecular genetic methods used are described in detail in these papers and will therefore not be commented on further here. Chromosome abnormalities were described according to the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN, 2005).

The aCGH method is a recently developed method which substantially contributes to the results and conclusions of three papers in this thesis and I will therefore make a few comments about the aCGH platform chosen for these studies.

There are several different aCGH platforms available, each differing in for example probe design and spatial resolution (Coe et al., 2007; Pinkel and Albertson, 2005). The reasons why we decided to use the oligonucleotide arrays instead of BAC-arrays were that the former allow immediate identification of the genes affected by genomic imbalances, and they also often have a higher resolution compared to many BAC-arrays.

The aCGH platform used in this thesis (Papers II-IV) is high-resolution 44K and 244K 60-mer oligonucleotide arrays containing approximately 43,000 and 236,000 probes, respectively (Agilent Technologies Inc.). These arrays have an average spatial resolution of about 35 kb and 6.4 kb, respectively, allowing detection of aberrations at the subgene level (Papers II-IV). The 244K platform has also demonstrated the highest sensitivitiy of the oligonucleotide platforms in a comparative study (Coe et al., 2007).

Although there are tremendous advantages with these new aCGH techniques one should bear in mind that they also have important limitations. Thus, balanced rearrangements such as reciprocal translocations and inversions, are impossible to detect (Shaffer and Bejjani; 2004). This calls for a combined strategy using multiple techniques to identify and in detail characterize genomic rearrangements in tumor cells.

Results and Discussion

***CHCHD7-PLAG1* and *TCEA1-PLAG1* gene fusions are generated by cryptic, intrachromsomal 8q rearrangements in PA (Paper I)**

Previous studies have suggested that cytogenetically cryptic gene fusions involving both *PLAG1* and *HMGA2* may occur in PA (Åström et al., 1999; Geurts et al., 1998; Stenman, 2005). The frequency of such gene fusions in PA is, however, unknown. To identify new fusion partners of *PLAG1* and to further study the mechanisms by which *PLAG1* fusions are generated, we analyzed a previously identified ectopic sequence fused to *PLAG1* in a PA with a t(8;15)(q12;q14) (Kas et al., 1997). This sequence was originally mapped to 15q14. Detailed analyses of publicly available sequence databases now revealed that the sequence is in fact derived from the first exon of the newly identified *CHCHD7* gene telomeric to *PLAG1*. RT-PCR and nucleotide sequence analyses of the PA with t(8;15) using primers located in exon 1 of *CHCHD7* and in exon 4 of *PLAG1* revealed chimeric transcripts containing exon 1 of *CHCHD7* fused to either exons 3 and 4 of *PLAG1* or to exons 2 to 4 of *PLAG1*. We further analyzed a series of 26 cytogenetically characterized PAs without known *PLAG1* fusions to search for additional tumors with *CHCHD7-PLAG1* fusions. RT-PCR analysis revealed *CHCHD7-PLAG1* fusion transcripts in two additional tumors, one with a t(6;8)(p21.3-22;q13) translocation and one with a normal karyotype.

Previous mapping studies of the t(6;8)-positive PA (Röijer et al., 1999) showed that the 8q translocation breakpoint is located approximately 20 megabases distal to *PLAG1* and *CHCHD7*. The corresponding breakpoint in the tumor with the t(8;15) maps at least 150 kilobases distal to *PLAG1*. Thus, both these tumors have 8q translocation breakpoints located clearly distal to *PLAG1,* as well as cytogenetically cryptic rearrangements joining the non-coding 5´-part of *CHCHD7* to the entire coding sequence of *PLAG1*. Based on their physical locations, a fusion between *CHCHD7* and *PLAG1* can be generated by a simple, paracentric inversion, resulting in promoter swapping between the two genes.

Upregulation of PLAG1 protein expression in the tumors was confirmed by Western blot and immunohistochemical (IHC) analyses. Northern blot and RT-PCR analyses revealed that, in contrast to *PLAG1*, *CHCHD7* is ubiquitously expressed, with the highest expression levels noted in adult tissues including normal salivary gland tissue.

CHCHD7 is a newly identified member of a multifamily of proteins with strong conservation at the structural level and low conservation at the amino acid level. Proteins in this family all contain a conserved (coiled coil 1)-(helix 1)-(coiled coil 2)-(helix 2) domain (Westerman et al., 2004). Another member of this subfamily, CHCHD1, was recently shown to be a nuclear protein expressed in human proliferative cytotrophoblasts and JEG3 choriocarcinoma cells but not in adult tissues (Westerman et al., 2004). The biological functions of CHCHD1 and CHCHD7 are unknown. Neither of these genes have hitherto been associated with neoplasia. Recently, Chchd3, a novel mitochondrial protein in mice, was identified as a substrate to the cAMP-dependent protein kinase (PKA), a kinase expressed in

mammalian cells playing a major role in metabolic control, growth and apoptosis (Schauble et al., 2007). Interestingly, another member of this multifamily of proteins, MTCP1, is involved in a gene fusion in mature T-cell proliferations, including T-cell prolymphocytic leukemias with t(X;14)(q28;q11) translocations (Fisch et al., 1993; Stern et al., 1993). MTCP1 is a mitochondrial protein that is overexpressed in leukemic cells due to fusions with the T-cell receptor alpha/delta locus (Soulier et al., 1994; Madani et al., 1995).

We have previously identified a recurrent and apparently cryptic *SII-PLAG1* fusion in PA (Åström et al., 1999). Studies have shown that *SII* is an intronless gene mapping to 3p22-p21.3 (Park et al., 1994; DiMarco et al., 1996). However, a new search of the NCBI databases now reveals that the *SII* sequence fused to *PLAG1* is in fact derived from exon 1 of the *TCEA1* gene located at 8q11.2 and that *SII*, now designated *TCEA1P2*, is a pseudogene. The breakpoint in *TCEA1* occurs in intron 1, resulting in a fusion of exon 1 of *TCEA1* to exon 2 of *PLAG1*. *TCEA1* has the same transcriptional orientation as *PLAG1* and is located about 2 Mb centromeric to *PLAG1*.

With this new information, we wanted to further investigate whether the *TCEA1-PLAG1* fusion also resulted from a cryptic, intrachromosomal rearrangement similar to the *CHCHD7-PLAG1* fusion. In order to do this, we isolated BAC clones containing the *PLAG1* and *TCEA1* genes as well as their 3′- and 5′-flanking sequences. Dual-color FISH analyses of interphase nuclei and nuclear chromatin fibers of a fusion-positive PA revealed that the two genes were indeed located close to each other and that the tumor had a *TCEA1-PLAG1* fusion. The transcriptional orientation of the *PLAG1* and *TCEA1* genes suggests that the fusion may be generated by an insertion of exon 1 and 5′-flanking sequences of *TCEA1* into intron 1 of *PLAG1*. Similar to tumors with *CTNNB1-PLAG1*, *LIFR-PLAG1* and *CHCHD7-PLAG1* fusions, the *TCEA1-PLAG1* fusions result in promoter swapping/substitution, leading to activation of *PLAG1*. Overexpression of the PLAG1 protein expression was confirmed using IHC. The majority of epithelial, myoepithelial, and mesenchymal-like cells stained positive for PLAG1, indicating that PLAG1 activation is a basic event that occurs early during the transformation of a putative pluripotent progenitor cell.

The fact that both the *CHCHD7-PLAG1* and *TCEA1-PLAG1* fusions are generated by cytogenetically cryptic rearrangements in tumors with different karyotypic abnormalities or normal karyotypes indicates that *PLAG1* fusions are more common than originally suggested by conventional cytogenetics. This assumption is also in line with previous molecular studies showing that ectopic expression of *PLAG1* may not only result from promoter swapping/substitution (Åström et al., 1999). Taken together, these studies demonstrate that activation of the *PLAG1* oncogene is a frequent and major genetic event occurring in all major cytogenetic subgroups of pleomorphic adenomas.

Cryptic gene fusions have recently also been found in several other types of neoplasms, such as leukemias (Barbouti et al., 2003; Graux et al., 2004; Paulsson et al., 2006), salivary gland mucoepidermoid carcinomas (Behboudi et al., 2006), sarcomas (Lestou et al., 2002; Sjögren et al., 2003), and prostate cancer (Tomlins et al., 2005, 2006). Together with the present results, these and other studies (Mitelman et al.,

2004) indicate that fusion genes may be much more common in human neoplasms, particularly in epithelial tumors, than previously anticipated.

Fusion and amplification of the *FGFR1* and *PLAG1* genes in ring chromosomes in PA (Paper II)

A subgroup of PA corresponding to approximately 4% are characterized by ring chromosomes of uncertain derivation. Here we have used SKY, FISH, and high-resolution aCGH to determine the origin and genomic content of these rings and to identify genes disrupted as a result of ring formation. A total of 16 tumors were analyzed regarding the derivation and genetic content of the rings. SKY analysis revealed that 11 tumors had rings derived from chromosome 8 and four of these had 2-4 r(8). The aCGH and FISH analyses revealed that these rings consisted of amplification of the pericentromeric segment 8p12□q12.1. The 44K arrays suggested that the breakpoints were close to the *FGFR1* and *PLAG1* genes in 8p12 and 8q12, respectively. Analysis of the 244K arrays indeed confirmed that the breakpoints had occurred within the 5´-parts of the *FGFR1* and *PLAG1* genes suggesting a possible fusion between these two genes as a result of the formation of r(8). The transcriptional orientation of the two genes are also in line with this reasoning. RT-PCR analysis confirmed that 9 of the 10 cases expressed *FGFR1-PLAG1* fusion transcripts. Seven tumors expressed chimeric transcripts containing exon 1 of *FGFR1* fused to either exons 2 or 3 of *PLAG1*, and two tumors expressed chimeric transcripts consisting of exon 2 of *FGFR1* fused to either exons 2 or 3 of *PLAG1*. FISH analysis revealed multiple *PLAG1* and alpha satellite signals on all r(8). The sizes of the rings correlated well with the number of *PLAG1* and alpha satellite signals in each ring.

Since the breakpoints in *FGFR1* and *PLAG1* occurred in the 5´-noncoding regions of both genes the major molecular consequence of this fusion is likely to be activation of *PLAG1* expression due to promoter substitution. Indeed, overexpression of *PLAG1* relative to normal salivary gland tissue was confirmed using real-time quantitative PCR and IHC analyses of fusion-positive tumors. The expression levels were equal or higher in tumors with r(8) and *FGFR1-PLAG1* fusions compared to tumors with *CTNNB1-PLAG1* fusions.

Copy number gains or amplifications of gene fusions are very rare and have previously only been observed in a few types of neoplasms, including chronic myeloid leukemia with *BCR-ABL* fusions (Johansson et al., 2002; Gorre et al., 2001), T-cell acute lymphoblastic leukemia with *NUP214-ABL1* fusions (Graux et al., 2004), and dermatofibrosarcoma protuberans with *COL1A1-PDGFB* fusions (Abbott et al., 2006; Kaur et al., 2006). Interestingly, these studies have suggested that amplification of fusion genes may indicate progression towards a more malignant phenotype and an aggressive clinical course. In line with these observations, we suggest that PAs with genomic instability and *FGFR1-PLAG1* gene fusions and/or copy number gain of *PLAG1* may be at increased risk of malignant transformation to Ca-ex-PA. This assumption is supported by previous findings of a Ca-ex-PA with amplification of *PLAG1* (Tsang et al., 2004) and with recent studies (Paper III) showing that 50% of Ca-ex-PA have copy number gains of a 1.4 Mb minimal common region in 8q12.1

including the *PLAG1* gene. Further, *PLAG1* has been found frequently amplified in hepatoblastomas, which are malignant, pediatric liver tumors (Zatkova et al., 2004).
FGFR1 has previously been implicated in several other neoplasms. For instance, gene fusions are found in which various N-terminal partners are fused to the tyrosine kinase domain of FGFR1 as a result of chromosomal translocations in the myeloproliferative syndrome (EMS)/stem cell leukemia-lymphoma syndrome (Xiao et al., 1998; Roumiantsev et al., 2004). Moreover, previous studies have shown that amplifications of at least four different regions within 8p11-12, including the *FGFR1* gene, are found in 10-15% of breast cancers (Ugolini et al., 1999, Gelsi-Boyer et al., 2005; Reis-Filho et al., 2006; Yang et al., 2006), and are associated with poor prognosis (Cuny et al., 2000; Chin et al., 2006). Similarly, amplifications of *FGFR1* and other genes within the proximal part of 8p are found in for example colorectal cancer (Nakao et al., 2004), urinary bladder cancer (Simon et al., 2001) and oral squamous cell carcinoma (Freier et al., 2007).
The present and previous studies illustrate the complexity of the rearrangements affecting 8p, with complicated patterns of amplifications and deletions, and of translocations resulting in gene fusions and disrupted genes in both hematological and solid neoplasms. The pattern and complexity of the rearrangements suggest that this is an inherently unstable genomic region that is prone to breakage, and that several genes in this region, including *FGFR1*, are important targets in various tumor types. It remains, however, to be shown whether gene fusions involving *FGFR1* might be found also in for example breast and colorectal cancers with translocation breakpoints and amplifications of 8p12.
One tumor with r(8) was negative for the *FGFR1-PLAG1* fusion. This case had instead gain of 8q10-q21.11 with at least two copies of *PLAG1* per ring and is therefore equivalent to cases with trisomi 8. The fact that the tumors with copy number gains overexpressed *PLAG1* at similar levels as tumors with *PLAG1*-fusions, demonstrates that copy number gain of an apparently normal *PLAG1* gene is an alternative mechanism for activation of this gene.
Interestingly, in six of the present tumors with ring chromosomes we discovered submicroscopic deletions of one or more regions within 3p21.3. Malignancy-related deletions involving this region have previously been found in many epithelial tumor types but also in many lymphoid malignancies (Kok et al., 1997; Kost-Alimova et al., 2003; Kost-Alimova and Imreh, 2007). Extensive experimental and clinical data suggest that this region harbors one or more tumor suppressor genes that when lost contribute to a malignant phenotype (Kost-Alimova et al., 2003; Kost-Alimova and Imreh, 2007). One can therefore speculate that deletions within 3p21.3 could be a genetic event that contributes to malignant transformation of pleomorphic adenomas.
We also identified tumors with ring chromosomes derived from chromosomes 1, 5, 6 and 9. Two tumors with r(5) had similar, but not identical, breakpoints in 5p14.1 and 5q21.1. The ring formation in these cases, however, did not result in any gain of chromosome 5 material. Nor did the breakpoints indicate that the formation of the rings could have resulted in gene fusions. Similarly, aCGH analysis of the breakpoints in the r(6) and r(9) did not indicate that gene fusions might have been generated as a result of the formation of these rings. Rather, our findings suggest that

these rings could result in loss of one or more putative tumor suppressor genes located distal to the breakpoints on the short and long arms of chromosomes 5, 6 and 9.

High resolution aCGH analysis of the 12q amplicons in PA and Ca-ex-PA identifies *HMGA2* as the single target gene (Paper III)

Cytogenetic evidence of gene amplification in the form of dmin and/or hsr were found in six of six PA and in two of 10 Ca-ex-PA. SKY analysis of dmin-positive tumors revealed that the dmin originated from chromosome 12.

To identify the genes amplified in dmin and hsr and to study possible differences in genomic imbalances between PA and Ca-ex-PA we performed aCGH analysis of five PA and 10 Ca-ex-PA. At least 13 recurrent copy number alterations were found in three or more cases each. The number of genomic imbalances per tumor was significantly higher in Ca-ex-PA compared with benign PA (24.5 vs. 3.8).

The most prominent finding in both PA and Ca-ex-PA was amplification (8 tumors) or gain (one tumor) of partly overlapping segments within 12q13.11-q21.3. Detailed analysis of the 12q amplicons revealed that the minimal common region, amplified in all cases, was a segment of less than 30 kb, including the first three exons (encoding the DNA-binding domains) of *HMGA2*. Several tumors had distinct breakpoints within *HMGA2* and immediately centromeric to the gene. Other recurrently amplified/gained genes were *MDM2*, *WIF1*, *TSPAN31*/*SAS*, *CDK4*, *OS9* and *GLI1*. FISH analysis of tumors with dmin revealed that the amplified 12q sequences exclusively mapped to the dmin.

Interestingly, four of the tumors showed breakpoints in both the *HMGA2* and *WIF1* genes, which are located only 0.7 Mb apart. Selective amplification of the 5´-part of *HMGA2* and the 3´-part of *WIF1* suggested that a possible fusion might have occurred between these two genes (Queimado et al., 2007). RT-PCR analysis indeed confirmed the expression of *HMGA2*-*WIF1* fusion transcripts in five of the 14 tumors analyzed. These findings demonstrate that *WIF1* is a recurrent and possibly frequent fusion partner of *HMGA2* in PA and Ca-ex-PA.

The *HMGA2* alterations found in the present tumors were very complicated. At least four different types of alterations of *HMGA2* were found, including amplification or gain of an apparently intact *HMGA2* gene, disruption of *HMGA2* (breakpoints in intron 3 or in the 3´-UTR) with amplification of the 5´-part or the entire coding sequence, and *HMGA2*-*WIF1* gene fusions with and without amplification of the fusion gene.

The consequences of amplification (and/or gene fusion) on the expression of *HMGA2* and several other genes, were examined by real-time quantitative PCR on 14 tumors. Irrespective of whether *HMGA2* was amplified, gained and/or involved in a gene fusion with or without amplification, the tumors showed very similar expression levels that were significantly higher compared to normal salivary gland tissue and tumors without *HMGA2* activation. There were no differences in expression levels between benign PA and Ca-ex-PA. High levels of HMGA2 protein expression was also confirmed by IHC. Our findings show that amplification, in

addition to gene fusion, is a novel mechanism of activation of *HMGA2* and that *HMGA2* is the single target gene of the 12q amplifications in PA and Ca-ex-PA.
HMGA2 has previously been shown shown to be the target gene in PA (Geurts et al., 1997a, 1998) as well as in benign mesenchymal tumors with translocations involving 12q13-15 (references in Stenman, 2005). *HMGA2* may also be amplified in for example bone and soft tissue sarcomas and gliomas (Berner et al., 1997; Reifenberger et al., 1996).
The present as well as previous investigations suggest that benign PA with amplification and overexpression of *HMGA2* and other 12q genes may have an increased risk of malignant transformation (Röijer et al., 2002; Rao et al., 1998). This assumption is in line with previous studies of oral squamous cell carcinomas, lung cancers, and pancreatic carcinomas, showing that high expression levels of *HMGA2* correlate with malignancy and poor prognosis (Miyazawa et al., 2004; Meyer et al., 2007; Abe et al., 2003). We suggest that the increased risk of malignant transformation of PA may also be due to genomic instability and to co-amplification of other cancer associated genes in 12q such as for example *MDM2*. In line with this suggestion, *MDM2* was overexpressed in all tumors with amplification.
The role of other frequently amplified genes such as *CDK4*, *OS9*, *XRCC6BP1*, *RAP1B*, *FRS2*, and *YEATS4* is uncertain. There were no substantial differences in expression levels between tumors with and without amplification of these genes. However, one cannot rule out that one or more of these genes could contribute to tumorigenesis in single cells.

Other recurrent aberrations were deletions of 5q, gains of two regions of 8q and amplification/gain of 17q21. Amplifications of 12q and loss of 5q23.2-q31 were the only aberrations found in both PA and Ca-ex-PA. Deletions of a 12.1 Mb common region in 5q23.2-q31.2 were found in 44% of the present tumors (two PA and five Ca-ex-PA). This region, which contains at least 87 genes, largely overlaps with a commonly deleted region in myelodysplastic syndromes and acute myeloid leukemia (Lai et al., 2001; Hejlik et al., 1997). Several genes in this region, including *SMAD5*, *KLHL3*, *MYOT*, *KIF20A*, *CDC23*, *CDC25C*, *JMJD1B*, *EGR1*, and *CTNNA1* have been shown or suggested to have a tumor suppressor function (Zavadil et al., 1997; Hejlik et al., 1997; Lai et al., 2001; Godley et al., 1999; Zhao et al., 1998; Horrigan et al., 2000; Hu et al., 2001; Liu et al., 2007). Collectively, the present and previous findings suggest that a tumor suppressor gene of importance for malignant transformation of PA is located within this region. Whether any of the above-mentioned genes implicated in myeloid malignancies also is the target gene of the 5q deletions in salivary gland tumors remains, however, to be determined.
Gain of a 1.4 Mb segment in 8q12.1 was identified in five of 10 Ca-ex-PA. This region only contains five genes of which *PLAG1* is the most obvious target gene (Stenman, 2005). This observation together with findings in Paper II suggest that copy number gain of *PLAG1* may contribute to malignant transformation of PA.

We also identified a 32.5 Mb segment in 8q22.1-q24.1, that was gained in seven of the ten cases of Ca-ex-PA. Of the 108 genes located in this region, *MYC* is a conceivable candidate gene. Amplification of *MYC* has previously been found in at least two other cases of Ca-ex-PA and never in benign PA (Rao et al., 1998; Tsang et al., 2004), indicating that this is likely to be a malignancy associated abnormality.

Similarly, we found amplification/gain of *ERBB2* in three cases of Ca-ex-PA. *ERBB2* encodes a growth factor receptor with tyrosine-kinase activity that is amplified and overexpressed in subsets of breast cancer and ovarian cancer (Slamon et al., 1989). Previous studies have also demonstrated preferential amplification and overexpression of *ERBB2* in malignant salivary gland tumors, including several cases of intracapsular Ca-ex-PA (Stenman et al., 1991; Di Palma et al., 2005). Available data indicate that *ERBB2* amplification is likely to be an early step in the malignant transformation of PA. Patients with ERBB2 positive tumors may also be potential candidates for treatment with trastuzumab (Herceptin).
Analysis of clinical follow-up data revealed that patients with 12q amplifications were substantially younger than those without 12q amplifications (median 69.5 years vs. 87 years) and they also had a much more favourable clinical outcome. None of them developed recurrencies or metastases during a mean follow-up of 8 years. In addition, our findings suggest that gains of 8q22.1-q24.1 (*MYC*), 8q12.1 (*PLAG1*), 1q11-q32.1, and 20 and loss of 1p21.3-p21.1 are associated with recurrent/metastatic disease. It should, however, be noted that the number of tumors analyzed is limited and that the results therefore have to be confirmed in larger investigations.
Taken together, our findings indicate that amplification and overexpression of genes in 12q including *HMGA2* and *MDM2*, deletions of 5q23.2-q31.2, gains of 8q12.1 (*PLAG1*) and 8q22.1-q24.1 (*MYC*), and amplification of *ERBB2* are genomic alterations of importance for malignant transformation of benign PA.

Detailed analyses of the 12q amplicons of a novel WDLS cell line (Paper IV)
In this paper we have genetically characterized a novel cell line designated GOT3, which was established from a retroperitoneal WDLS. Cytogenetic analysis of primary cultures of the first recurrence of the WDLS revealed the following pseudodiploid karyotype 46,XX, -1, t(1;4)(q12;q13), -14, der(16)t(7;16)(p11.1;p13.3) dup(16)(q11.2q12.1), +20, +mar. Repeated analysis of a later passage of the GOT3 cells showed that they had retained the giant marker. SKY analysis of these cells confirmed the t(1;4) and the der(16) rearrangements and showed that the giant marker was composed of material from chromosomes 1, 12 and X. This is in agreement with previous studies showing that giant markers in WDLS mainly consist of chromosome 12 material, but may also contain sequences derived from chromosome 1 (Pedeutour et al., 1994, 1999; Gisselsson et al., 1998; Kresse et al., 2005; Mitelman et al., 2007a).
To our knowledge, this is the first array-based oligonucleotide CGH analysis of WDLS. Analysis of both the 44K and 244K arrays revealed that copy number gains were three times more common than losses. A total of 23 gains and 8 losses were identified. Based on SKY and aCGH the giant marker typical of WDLS was composed of amplified sequences derived from 12q11-q21.2, 1q23.3-q44, and Xp22.2-p22.31 as well as of a single copy of 1p31.1-pter.
Detailed analysis of the 12q amplifications revealed that 84 of the 144 amplified genes were highly amplified. The genes with the highest level of amplification were *MDM2*, *HMGA2 (5´-part)*, *CDK4*, *SAS*/*TSPAN31*, *GLI*, and *DDIT3*. Previous studies of WDLS have shown that *MDM2* and *CDK4* are highly amplified in most cases and

that *SAS*/*TSPAN31* and *HMGA2* are co-amplified less frequently (Pedeutour et al., 1994, 1999). Of these genes, *MDM2* is considered as the major target gene. In contrast, *HMGA2* is the target gene of copy number alterations and rearrangements/gene fusions in benign lipomas with 12q13-15 aberrations (Italiano et al., 2007; Bartuma et al., 2007). Interestingly, there are also a few examples of WDLS cases with 12q amplification in which ectopic sequences from for example 1q24 are fused to *HMGA2* (Meza-Zepeda et al., 2001), indicating a possible link between benign lipomas and WDLS.

To study the consequences of gene amplification on gene expression we performed real-time quantitative PCR analyses of 10 genes in 12q, four genes in 1q, and one gene in 13q. All highly amplified genes were overexpressed in GOT3 cells relative to normal, control fibroblasts. *HMGA2* was the most highly expressed gene. Using IHC and Western blot we could also show that the HMGA2 protein was overexpressed compared to normal fibroblasts. Our aCGH findings of rearrangements of *HMGA2* with amplification of the 5´-part of the gene, including the part encoding the three DNA-binding domains, together with the expression data, clearly demonstrate that *HMGA2* indeed is activated and overexpressed in the GOT3 cells and therefore is likely to contribute to the pathogenesis of WDLS. As shown in Paper III, *HMGA2*, and somewhat less frequently also *MDM2*, *CDK4*, *SAS*/*TSPAN31* and others, are highly amplified in subsets of PA and Ca-ex-PA, and that benign tumors with rearrangements/amplification of *HMGA2* are prone to malignant transformation (Röijer et al., 2002; Paper III).

Within the 12q amplicons we also identified several highly amplified genes previously not implicated in WDLS, including for example *OS9*, *XRCC6BP*, *WIF1*, *RAP1B*, *YEATS4*, *FRS2*, *TSPAN8*, and *CSRP2*. The majority of these genes encode proteins of potential or proved cancer relevance. For example, *YEATS4* is involved in transcriptional activation and was recently shown to repress the *TP53* tumor suppressor pathway (Park and Roeder, 2006; Llanos et al., 2006). Interestingly, *MDM2*, which was among the most highly amplified and overexpressed genes in GOT3, is also known to inactivate *TP53*. Since no expression of TP53 was observed in the primary tumor or in the GOT3 cells, one may speculate that amplification and overexpression of *MDM2* and *YEATS4* may contribute to *TP53* down-regulation.

In addition to the major amplifications in 12q13-15, we also detected high level amplifications in 1q23.3-q44. Of the genes in this region, *DNM3* showed the highest level of amplification followed by *KIF21B*, *FASLG*, *CACNA1S*, *CSRP1*, *TMEM9*, *IPO9*, *PLA2G4A*, *PKP1*, and *HIST3H2A*. Interestingly, a previous study have described a case of dedifferentiated retroperitoneal WDLS in which gain of 1q22-q24 was associated with lung metastases (Forus et al., 2001). This segment of 1q, which partly overlaps with the 1q amplicons in the GOT3 cells, may thus contain genes that when amplified and overexpressed can contribute to a malignant phenotype.

The 12q13-15 region is the target of amplifications in many types of benign and malignant neoplasms, including soft-tissue sarcomas (Berner et al., 1997; Pedeutour et al., 1994, 1999; Gisselsson et al., 1998; Fritz et al., 2002; Coindre et al., 2004; Heidenblad et al., 2006), osteosarcomas (Berner et al., 1997; Ladanyi et al., 1993; Gisselsson et al., 1998, 2002; Atiye et al., 2005; Heidenblad et al., 2006), Ewing's

sarcomas (Ladanyi et al., 1995), neuroblastomas (Corvi et al., 1995), breast carcinomas (Quesnel et al., 1994; Courjal et al., 1996; Cuny et al., 2000; Al-Kuraya et al., 2004), gliomas (Reifenberger et al., 1994, 1996; Fischer et al., 1996), urinary bladder cancer (Simon et al., 2002), prolactinomas (Finelli et al., 2002), PA, and Ca-ex-PA (Röijer et al., 2002; Paper III). Thus, subsets of tumors originating from a variety of cell types, such as mesenchymal, neuroectodermal, neuroepithelial, and epithelial, have amplification of genes derived from the same 12q region. The amplified genes may be cytogenetically visible as dmin, hsr, giant markers, or ring chromosomes. Available data also indicate that the target genes of these amplifications may be different in different tumors. In the majority of the above-mentioned tumor types, however, *MDM2* and *CDK4* have been suggested as the major targets. In contrast, the present studies and those of prolactinomas for example, identify *HMGA2* as the target gene. The identification of target genes is complicated by the fact that many studies have only analyzed selected candidate genes and have not performed genome-wide aCGH analysis. In order to identify novel targets, and to get a complete and detailed picture of the amplicons, large genome-wide, high-resolution aCGH studies have to be performed on these tumor types. Our findings in Papers II-IV show that this may be a rewarding approach.

Conclusions

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

- *CHCHD7-PLAG1* and *TCEA1-PLAG1* are novel gene fusions generated by cryptic, intrachromosomal 8q12 rearrangements in PA.

- PA with ring chromosomes derived from chromosome 8 contain amplification of a pericentromeric segment with breakpoints in *FGFR1* in 8p12 and in *PLAG1* in 8q12.1, resulting in novel *FGFR1-PLAG1* gene fusions. An alternative mechanism of *PLAG1* activation is copy number gain of an intact *PLAG1* gene. The results further illustrate the versatility of the *FGFR1* and *PLAG1* genes in tumorigenesis.

- *HMGA2* is the major target gene of 12q amplifications in PA and Ca-ex-PA. *MDM2* is co-amplified in 90% of the cases. Tumors with amplification of *HMGA2* may have cryptic *HMGA2-WIF1* gene fusions.

- Ca-ex-PA display a significantly higher number of genomic imbalances compared to benign PA (24.5 vs. 3.8).

- Amplification of *HMGA2* and *MDM2*, deletions of 5q23.2-q31.2, gains of 8q12.1 (*PLAG1*) and 8q22.1-q24.1 (*MYC*), and amplification of *ERBB2* are genetic alterations that may contribute to malignant transformation of benign PA.

- Cryptic gene fusions involving *PLAG1* and *HMGA2* are more common in PA and Ca-ex-PA than previously anticipated.

- *HMGA2* and *MDM2* are major targets of the 12q amplifications in giant marker chromosomes in WDLS.

- High-resolution, oligonucleotide aCGH is a very useful tool for detecting genomic imbalances, mapping translocation breakpoints, and identifying novel fusion oncogenes.

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