A Thesis entitled

The Mechanisms of Malignant Transformation in Benign Salivary Gland Tumors

Submitted by
Yasmyne S. Castillo-Ronquillo MD

As partial fulfillment of the requirements for the Master of Science in Biology

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Adviser: Patricia Komuniecki Ph.D.

________________________________________________________________________
College of Graduate Studies

The University of Toledo
August 2009
An Abstract

of

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Tumors of the salivary glands are some of the most complex tumors known. Although the progression from a benign salivary gland tumor to a malignancy has been documented in the literature, this process is not well understood. Pleomorphic adenoma (PA) is a type of benign tumor known clinically and histopathologically to transform into a malignant form in both the salivary glands and lacrimal glands. Pleomorphic Adenoma Gene 1 (PLAG1) overexpression is the initial abnormality found in PA. The molecular changes in the progression from PA to early stages of malignancy have not been fully elucidated. However, the inactivation of tumor suppressor genes and the activation of oncogenes and proto-oncogenes appear to be involved in the early transition phase to malignancy. The inactivation of p53, the loss of DCC, p16 and the activation of the oncogenes p21, c-myc and c-ras have
been documented in cell culture, animal studies and human salivary gland tumors. In the intermediate and late stage of the transformation of PA to a malignant carcinoma ex pleomorphic adenoma (CXPA), the cell cycle genes CDC25A, erb-2, cdk-4, E2F-1, Bub-1, STAT3 are involved. The receptors PDGFR, Her-2/neu, androgen receptor and BRST-2 receptor are upregulated in some malignant tumors of the salivary gland. A model for the malignant transformation of pleomorphic adenoma to carcinoma is proposed. This model is analogous to the multi-stage tumorigenesis model of colon cancer. The studies of salivary gland tumors can serve as templates for the study of the malignant transformation in lacrimal gland tumors, the literature of which is sparse.
Acknowledgements

I would like to acknowledge and thank Dr. Patricia Komuniecki, who has continuously inspired me to pursue MS studies in biology and has found time in her immensely busy schedule to edit this paper; Dr. Song-Tau Liu who has patiently guided me in the laboratory; Dr. Lirim Shemshedini and Dr. Leisner, for their inspiring lectures in my first semester of graduate training; Dr. William Taylor, for accepting the task of reading and commenting on my manuscript as a member of the committee; Dr. Diakanova, who presented exciting videos of the cell machinery; Dr. Bamber and Dr. Leaman, who painstakingly explained the details of laboratory procedures which were new to me, having been once trained as a chemist; Dr. Guofa Liu, for his incisive comments on the neuroscientific literature; Dr. Fan Dong and his staff for allowing me to use their lab equipment; Aaron Tipton for his guidance in the lab; Adam Pore, who was my tutor par excellence in research methods; all the graduate students, whose research presentations have always amazed me; Dr. Richard Komuniecki, for his invaluable comments during the presentations; the faculty of the biological sciences department who were always available to answer questions; Linda and Carol, who helped me navigate the graduate school system; Director Manuel Agulto MD and Professor Milagros Leaño of the University of the Philippines Manila for their continued support; Dr. Ralph Valenzuela of the Institute of Ophthalmology for the histopathology slides and my sons, Nikko and Micah, for their encouragement and technical help in the formatting of the manuscript.
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Abbreviations

Tumors
ACC: adenoid cystic carcinoma
ACN: adenocarcinoma
CXPA: carcinoma ex pleomorphic adenoma
CPA: Carcinoma in pleomorphic adenoma
EMC: epithelial-myoepithelial carcinoma
HNCA: head and neck carcinoma
MEC: Mucoepidermoid carcinoma
MEM: Malignant myoepithelioma
MSE/MSA: established epithelial cell lines
NSG: Normal salivary glands
PA: pleomorphic adenoma
PLGA: Polymorphous low-grade adenocarcinoma
SGT: Salivary gland tumors

Chromosomes and Genes
FAL: Frequency of allelic loss
FAP: Familial adenoma polyposis
HER2: Human epidermal growth factor receptor
HMGA2: High Mobility Group A2
LOH: Loss of heterozygosity
MDM2: Mouse double minutes 2
MMTV: mouse mammary tumor virus
PLAG-A: Pleomorphic Adenoma Gene A
STAT3: Signal transducers and activators of transcription

Transcription Factors, Receptors, Proteins
AR: androgen receptor
BRCA: Breast Cancer Gene
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
FGF: Fibroblast Growth Factor
IGF: Insulin-like Growth Factor
LIFR: leukemia inhibitory factor receptor
PCNA: Proliferating cell nuclear antigen
PDGF: Platelet derived growth factor
PDGFR: Platelet derived growth factor receptor
TERT: Telomerase reverse transcriptase
VEGF: Vascular endothelial growth factor
XIAP: X-linked inhibitor of apoptosis protein

Cell Cycle Components
CDK: Cyclin dependent kinase
**Lab Techniques**

**MVD:** Microvessel diameter

**PCR:** polymerase chain reaction

**RT:** reverse transcriptase

**TVA:** Total microvascular area
I. Introduction

Malignant mixed tumors are one of the most complex tumors known. Mixed tumors may be benign or malignant and may appear in salivary glands, lacrimal glands and other glandular structures. Malignant tumors of the salivary glands are a rare oral neoplasia, comprising less than 0.5% of all malignancies combined and approximately 5% of malignancies of the head and neck region. These tumors are thought to develop de novo in the salivary gland and contain malignant stromal and epithelial elements. Malignant mixed tumors of the lacrimal gland are even rarer.

Three different subcategories of malignant mixed tumors have been defined: carcinosarcomas, carcinoma ex pleomorphic adenoma and metastasizing pleomorphic adenomas. Carcinoma ex pleomorphic adenoma (CXPA) arises from a pre-existing benign mixed tumor. The patient’s clinical history usually reveals a long-standing salivary gland lesion and the patient may have undergone multiple operations for recurrent pleomorphic adenoma. Most investigators have hypothesized that the carcinoma arises directly from de-differentiation or malignant transformation of the benign tumor. Several experimental models have been used to understand the malignant transformation in salivary glands. These include the use of chemicals, viruses, radioactive isotopes, and genetically engineered animals.

Although benign mixed tumors have been well known to degenerate into malignancy after many years, the mechanisms are still unclear. This review will discuss some of the most recent research on the possible mechanisms of malignant degeneration.
of salivary gland tumors. While rare, these tumors behave aggressively and the 5-year survival is poor. They are difficult to manage clinically. This paper will discuss possible mechanisms for malignant transformation based on a review of studies on tumors of the salivary glands and will apply these findings for future studies on the malignant transformation in lacrimal gland tumors.
II. Histopathology and Classification of Benign and Malignant Tumors of the Salivary Glands

Despite the complex cellular differentiation in the various salivary glands, there are two main types of cells: luminal and basal. The duct luminal cells, whether at the proximal or distal portions of the duct system or the acini, are associated with basal and/or myoepithelial cells and the acinar cells support myoepithelium.\textsuperscript{9}

In human salivary glands, there is no distinct separation of the duct system into intercalated, striated or excretory segments but rather, a gradual transition exists between them.\textsuperscript{10} Acinar cells differentiate as both serous and mucinous cells, with the secretory granules exhibiting a variety of ultrastructural appearances that differ between species and in different glands of the same species.\textsuperscript{11,12} Under certain circumstances, luminal cells have a range of differentiation capabilities. Acinar cells are capable of dedifferentiation to ductlike cells and squamous metaplasia.\textsuperscript{13} Basal or myoepithelial cells of human salivary glands are present on the outer aspect at all levels of the ducts as well as acinar units. Immunocytochemically, basal cells of excretory and striated ducts are heterogeneous with respect to their content of cytokeratins and muscle specific actin. Basal cells at any level of the duct system and myoepithelial cells of striated and intercalated ducts and acini share a complement of cytokeratin.\textsuperscript{13}

The hybrid nature of myoepithelium, a combination of epithelial and smooth muscle characteristics, suggests a contractile function. However, the full function of myoepithelial cells remains to be elucidated. Immunoelectron microscopy of the human
parotid reveals the complex interaction of actin and cytokeratins. The principal intermediate filaments of epithelial cells, the family of cytokeratins, serve as valuable lineage markers. Basal and myoepithelial cells express cytokeratin, much like basal cells of the epidermis and oral mucosa. 13-15

Some salivary gland tumors display an overlap in terms of cellular differentiation. For example, neoplastic myoepithelial cells in both adenoid cystic carcinoma and basal cell adenoma can participate in formation of glandular lumens. 16,17 This observation emphasizes the central role of tumor cell differentiation in these neoplasms, as it suggests that any one tumor cell has the potential to differentiate in at least two directions-ductal or myoepithelial/basal- or assume a hybrid form.9

Dardick and Van Nostrand (1985) reviewed the three potential mechanisms for the derivation of new secretory or other specialized cells of the duct system: 1) Replication or mitotic division of existing fully differentiated cells, 2) differentiation from a population of uncommitted stem cells or committed precursor cells, and 3) transformation or the process of metaplasia in which one type of specialized cell modifies to assume characteristics of another cell type. 9

Primarily, monocellular salivary gland tumors are composed either of luminal-type cells, with or without acinar differentiation or of the neoplastic counterpart of myoepithelial and /or basal cells. (Fig.1)
These models depict the three basic forms of salivary gland tumors: (1) involves differentiation of duct luminal cells plus basal-like and/or myoepithelial-like cells; (2) reveals only duct luminal and/or acinar cells; and (3) is composed solely of myoepithelial-type and/or basal-like tumor cells.

The presence or absence of significant amounts of specifically localized proteoglycans, basal lamina, other collagens and elastin in relation to the neoplastic myoepithelial/basal cells provides the other major criterion for classification. (Fig. 2)
In addition to the combinations of luminal and myo-epithelial/basal cell differentiation in these tumors, the type and extent of synthesis as well as the distribution of extracellular materials considerably influence the final histology of salivary gland tumors. The lower part of the above figure demonstrates focal or expanding domains of extracellular materials that develop among the myoepithelial/basal cell compartment of a stylized neoplasm.

In adenoid cystic carcinoma, well-controlled localization of proteoglycans and basal lamina occurs, whereas in pleomorphic adenoma excessive production forces
separation of adjacent tumor cells and the eventual development of myxochondroid zones. Dardick and Van Nostrand proposed a simple taxonomic outline to serve as a basis for defining diagnostic criteria for the complex array of neoplasms originating in these glands.  

Figure 3. Taxonomic classification of salivary gland tumors. (Dardick and van Nostrand 1988).

By analyzing the type or types of differentiated cells and their organization and synthetic products, it is possible to rearrange the multiplicity of salivary gland tumors in a more systematic way. The diagram illustrates how detection of the main differentiated cell or cell types and the presence or absence of basal lamina and glycosaminoglycans allows development of a list of neoplasms for differential diagnosis.

A review of the literature shows the wide diversity of benign and malignant tumors of the salivary glands depending on the cell type and stromal appearance of the
tumors. Among the multiplicity of histological features are cribriform patterns, ducto-glandular elements and myxoid stroma.

In general, benign mixed tumors are characterized by the presence of benign acinar cells, ducts and myxomatous matrix. Malignant mixed tumors are characterized by the abnormal proliferation of luminal cells and stroma. The histology of benign salivary glands as contrasted with the histopathology of pleomorphic adenoma and malignant mixed tumor of both the lacrimal gland and salivary glands are presented in the appendix. The representative micrographs were taken with permission from the files of the Institute of Ophthalmology and the College of Medicine, Department of Pathology, University of the Philippines Manila.
III. Chromosomal Aberrations, Oncogene Expression and Tumor Suppressor Gene Abnormalities in Human Salivary Gland Tumors

Chromosomal banding data on chromosomal aberrations and oncogene expression is available in 350 pleomorphic adenomas (PAs).18,19 These reports indicate that about 50% of PAs have a normal karyotype. Among the observed chromosomal defects are:

1. Translocations (infrequently deletions) involve chromosome 8 (band q12);
2. Deletions affect chromosome 3 (usually either 3p21 or 3q21-27); and

A number of oncogenes, such as c-erbA-2, c-raf, c-mos, c-int, and c-gli, not only map to these chromosomes, but also are in the proximity of bands involved in the breakpoints noted in the tumors. Certain cell cycle-related genes, such as CDC25A, map to the region of the chromosome 3 breakpoints noted in karyotypic abnormalities of some pleomorphic adenomas. Sandros and associates (1990) also karyotyped 33 human malignant salivary gland tumors, including adenoid cystic carcinomas (ACC), acinic cell carcinomas, mucoepidermoid carcinomas (MEC), adenocarcinomas, epidermoid and undifferentiated carcinomas. Unlike the benign lesions, the most consistent structural abnormality in the malignant tumors was a deletion involving the long arm of chromosome 6, usually in the q22-25 region. Four oncogenes have been localized to the distal portion of 6q: c-syn, c-ras, c-myb, and c-mas. These studies suggest that different
genes may be involved in the histogenesis of benign and malignant human salivary gland tumors.

Malignant mixed tumors of the salivary gland are diverse and include carcinomasarcomas, carcinoma ex pleomorphic adenoma (CXPA) and metastasizing PAs. Carcinosarcomas (true malignant mixed tumors) of the salivary glands are rare biphasic tumors that exhibit both carcinomatous and sarcomatous elements. CXPA arises from a pre-existing benign mixed tumor. Usually, the patient has a long-standing history of a salivary gland lesion, and may have undergone multiple operations for recurrent PA. Most of these tumors will have malignant epithelial components but not malignant stromal components. Based on the morphologic features and genetic analyses, investigators have hypothesized that the carcinoma arises directly from de-differentiation or malignant transformation of the benign tumor.

Fowler et al. (2006) examined a group of malignant mixed tumors, including both carcinomasarcomas and CXPAs of the salivary gland, using a loss of heterozygosity (LOH) analysis of a panel of tumor suppressor genes. They described a mutational profile for each morphologically unique tumor component, with direct comparison between the different compartments, as a tool to assess common clonal origin. DNA extraction of target areas of deparaffinized sections of tumors diagnosed as malignant mixed tumor of salivary gland origin was done. Then PCR was performed using a set of primers that amplify short tandem repeat units at or near known tumor suppressor genes. The ratio of the peak heights (allele ratio) was obtained for informative (heterozygous) markers in both the tumor and the normal tissues. The allele ratio for the tumor was divided by the allele ratio for normal tissue in order to assess for LOH. The fractional allelic loss (FAL)
was calculated as the number of chromosomal arms showing LOH divided by the total number of informative loci. Of the 13 different specimens arising in 11 different patients, there were six high grade carcinosarcomas, three recurrences of a low-grade carcinosarcoma and four CXPAs. The malignant tumor components analyzed at the molecular level all had a relatively high frequency of FAL with an average of 42% for all malignant targets examined. Allelic loss of 17q21 and 9p21 were only seen in carcinosarcomas and not in CXPAs. Allelic losses in three genetic loci occurred at high rates: 73% at 17p13, which is the location of the p53 gene, 55% at 17q at the location of the nm-23 gene and 50% at 18q at the location of the DCC gene. Few allelic losses were seen in the benign component and a significantly higher rate of allelic loss in the malignant areas of the CXPAs. The other tumor suppressor genes examined included VHL, hOGG1, APC, CDKN2 and DCC. The findings provide supporting evidence for the theory that all malignant elements in these tumors are derived from a common clonal precursor. The benign components of CXPAs do not show a high–level frequency of FAL. A common clonal origin was suggested in two out of four cases in the CXPAs supportive of previous observations. \textsuperscript{22,23}

Hybrid carcinomas of the salivary gland are a recently defined and rare tumor entity, consisting of two histologically distinct types of carcinoma within the same topographic area. Various carcinoma combinations have been described in hybrid carcinomas; salivary duct carcinoma, epithelial-myoepithelial carcinoma (EMC) and adenoid cystic carcinoma are frequently involved. Although little prognostic information is available, several investigators have suggested that the aggressiveness of hybrid carcinomas is determined by the histologically higher grade component. \textsuperscript{24-28}
In a study of nine cases of hybrid carcinomas of the salivary gland, a diffusely positive p53 immunoreactivity was found in four cases, and the positivity was restricted to the more aggressive component in each pair. Furthermore, p53 gene alteration analysis of the p53 positive cases revealed that all demonstrated LOH at p53 microsatellite loci and p53 gene point mutations. Thus, molecular genetic events may play an integral part in inducing the transformation from histologically lower to higher grade tumor during the hybrid carcinoma genesis of the salivary glands.\textsuperscript{29}

In pleomorphic adenomas, activation of the Pleomorphic Adenoma Gene 1 (PLAG1) on chromosome 8q12 is the most frequent mutation found. Since PLAG1 is a transcription factor, abnormal PLAG1 expression or activity results in deregulation of PLAG1 target genes, causing salivary gland tumorigenesis. Activation of the PLAG1 gene results from chromosomal translocations leading to promoter substitution between PLAG1 which is mainly expressed in fetal tissue, and more broadly expressed genes. The replacement of the PLAG1 promoter, inactive in adult salivary glands, by a strong promoter derived from the translocation partner, leads to ectopic expression of PLAG1 in the tumor cells. PLAG1 binds to promoter 3 of the Insulin-like growth factor 2 gene (IGF2) and stimulates its activity. IGF2 is highly expressed in salivary gland adenomas overexpressing PLAG1 while no IGF2 expression is found in adenoma without abnormal PLAG1 expression nor in normal salivary gland tissue.\textsuperscript{30}
The minimal PLAG1 binding site is composed of two essential parts, a CRGGC core separated by seven random nucleotides from an RGGK cluster. This bipartite binding site is quite unusual and can be explained by the particular way PLAG1 binds DNA. Two non-contiguous regions in PLAG1 are essential for DNA recognition: finger 3 interacting with the G-cluster and fingers 6 and 7 recognizing the CORE (Fig. 4a).

**Figure 4a. Model depicting the mode of interaction of PLAG1 with its consensus DNA binding site.** (Adapted from Voz ML 2001).  

Promoter swapping has been identified between the PLAG1 and β-catenin genes (CTNNB1) and results in up-regulation of PLAG1 expression and down-regulation of CTNNB1 expression.

A recurrent translocation t(5;8)(p13;q12) in PAs results in promoter substitution between the PLAG1 and the leukemia inhibitory factor receptor (LIFR) which has been previously mapped to 5p12-p13. Under the control of the LIFR promoter, ectopic expression of PLAG1 is observed in tumors, which probably leads to an abnormal expression of target genes causing tumorigenesis.
Figure 4b. Schematic representation of the promoter swapping between PLAG1 and the β-catenin genes and promoter substitution between PLAG1 and the LIFR genes. (Adapted from Voz ML et al. 2001). 31

One novel fusion partner of PLAG1 is the gene encoding the transcription elongation factor IIS (S-II). There could be other fusion partners of PLAG1 which have not been identified to date.
IV. Oncogene Expression in Animal Models and PLAG-1 Expression in Cell Lines

Animal models are now used to study the salivary gland cells involved in tumor initiation and the mechanisms responsible for the morphology of salivary gland neoplasms. Oncogene constructs using a variety of promoter/enhancer elements of different genes were inserted into mouse germline DNA in order to study mammary gland carcinogenesis. These have included mouse mammary tumor virus (mMTIV)/c-myc, mMTIV/v-Ha-ras, mouse whey acidic protein (wap) promoter/v-Has-ras and mMTV/c-neu. Most female transgenic ras mice express mRNA for ras oncogene in both mammary and salivary tissues. Some tumors develop in the parotid glands of transgenic mice, primarily males, carrying mMTV/v-Ha-ras. Male mice carrying the Ha-ras transgene driven by the wap promoter may develop tumors of the submandibular gland. Thus, the wap promoter may be specific for the submandibular gland whereas the mMTV promoter appears to be specific for the parotid glands. In MMTV-ras and wap-ras lines, both salivary gland and mammary gland tumors develop, suggesting similarities in the genetic or cellular environment of both glands. However, salivary gland tumors occur mainly in males, whereas the mammary tumors occur predominantly in females, suggesting that hormonal factors influence whether tumors develop in mammary or salivary glands.

Another form of transgenic mice, int-1 oncogene (mMTV LTR/c-int-1) mice, develop adenocarcinomas in the salivary glands of both male and females with high
levels of mRNA for this gene expressed in both mammary and salivary gland tissues. It is interesting to note that in all mice bearing the mMTV/v-Ha ras transgene that developed tumors, the development of palpable parotid lesions was preceded by enlargement of the intraorbital lacrimal gland, so that exophthalmos, whether unilateral or bilateral was a phenotypic marker for tumor development. Thus, these transgenic mice also can be used as a model for lacrimal gland anomalies.

Transgenic mouse models have used the mouse mammary tumor virus (MMTV) promoter to target the expression of different oncogenes to salivary and mammary glands. The resulting salivary gland phenotypes are not homogenous across different models and have been less characterized than mammary gland tumors. Leaky transgenic expression of oncogenes in salivary gland tissues when using a variety of promoters can result in a low incidence of salivary gland tumors. Among the promoters of H-ras oncogene used were murine wap promoter 1 and human prostate-specific antigen promoter 2. SV40 large T antigen expression using a promoter from the neonatal submandibular gland secretory protein b gene has also been reported to cause salivary gland tumors.

A model in which a ras oncogene is expressed in cytokeratin 5 (K5)-expressing cells on doxycycline administration was used to explore the effects of this oncogene in the salivary glands of adult mice. Inducible expression of a mutated K-ras gene under the control of the K5 promoter led to the development of hyperplastic and dysplastic epithelial lesions and carcinomas, with an incidence of 100% and a minimum latency of a week. The tumors appear to arise from the cytokeratin 5-positive basal cell compartment. Myoepithelial cells participated in the hyperplasias but not in carcinomas. Those
investigators reported that the ras oncogene, targeted to a specifically sensitive cell compartment within the salivary glands, can trigger a series of events that are sufficient for full carcinogenesis. Most proliferative lesions in the K5-tet-on tet-ras model seem to proceed through squamous metaplasia. It is possible that Ras may stimulate signal transduction pathways promoting squamous differentiation as part of the carcinogenic process. Proliferation is uncoupled from differentiation in this model, possibly accounting for the rapid development of carcinomas. Raimundi and Cross (2006) speculate that Ras expression in a compartment that includes the salivary gland stem cells is sufficient to trigger the acquisition of an oncogenic and metastatic phenotype. They hypothesize that there is no requirement for a multistep process involving the accumulation of mutations in the p53 family that can inhibit p53 function. Alternatively, ras may inhibit p53 function by a still not fully understood mechanism. In this study, the researchers also observed a highly elevated expression of the ΔN isoform of p63 in ras-induced salivary gland tumors as compared to normal and non-neoplastic tissues and this splice variant of p63 can inhibit p53 transcriptional activity.

Pleomorphic adenomas (PAs) of salivary glands are characterized by the mixed appearance of epithelial and mesenchymal-like components such as myxoid and chondroid tissues. Although various studies have examined PAs, thus far it is not clear how PAs make these multiple components. Thus, clarification of the histodifferentiation of this unique salivary gland tumor using not only tissues in vivo, but also PA cells cultured in vitro, is necessary. The first in vitro PA model was reported in 2007 by Kitagawa et al. Normal and benign tumor cells tend to grow slowly and senesce quickly in culture. Therefore, Kitagawa immortalized cells using transfection of the
hTERT gene without otherwise altering the nature of those cells. The immortalized PA cells expressed mRNA of the PLAG1 and showed epithelial and neoplastic myoepithelial characteristics by immunohistochemical immunofluorescence analyses and ultrastructural study. Their findings suggest that these cells will be a useful model to study the cellular differentiation of PA.
V. **Cell Cycle Regulators in Cell Culture and in situ Studies in Salivary Gland Tumors**

The expression of G1-phase cell cycle regulators is commonly deregulated in human malignancies. In a study by Etges et al.\(^{37}\), the expression of the retinoblastoma pathway proteins was investigated in normal salivary glands (NSG) and in salivary gland tumors (SGT) using immunohistochemical techniques. The salivary gland tumors included PA, adenoid cystic carcinoma (ACC), mucoepidermoid carcinoma (MEC), epithelial-myoepithelial carcinoma (EMC), CXPA, malignant myoepithelioma (MEM) and polymorphous, low-grade adenocarcinoma (PLGA). Antibodies to cyclin D1, cyclin-dependent kinase 4 (CDK-4), retinoblastoma protein (pRb), CDK inhibitor p16 and transcription factor E2F-1 were used. In normal salivary glands, cyclin D1 and cdk-4 were not expressed in any tumor cells, while p16 was positively expressed, pRb was abundant and E2F-1 moderately expressed. In tumors, CDK-4 was overexpressed in half of the cases. Most tumor cases showed decreased pRb immunoexpression compared to NSG. In contrast, expression of p16 and E2F-1 increased. pRB expression was absent in 3 cases of PA, one of CXPA and two of EMC. One case of MEM and one of PLGA showed no E2F-1 expression. Statistical analyses showed positive correlations between cyclin D1 and cdk-4, cyclin D1 and E2F-1, cdk-4 and E2F-1, and p16 and E2F-1. The benign and malignant tumors expressed retinoblastoma pathway proteins differently from the normal salivary gland.\(^{37}\) Table 1 summarizes the study of Etges\(^{37}\) and Patel\(^{43}\).
The cell cycle regulators such as human Bub1 play an important role at the spindle assembly check-point to prevent cell cycle progression following spindle damage. The spindle assembly check-point ensures the fidelity of chromosome segregation by delaying anaphase until all chromosomes are correctly attached to the spindle and increases the probability of successful delivery of an euploid chromosome set to each daughter cell. Bub1 encodes a protein kinase that localizes to the kinetochore and can phosphorylate Bub3 protein. In the presence of spindle damage, Bub1 inhibits the cell cycle progression into anaphase. Genetic alteration of the Bub1 gene rarely occurs in human cancers such as breast cancer, lung cancer and gastric carcinomas. Twenty-one human salivary gland tumors were investigated to determine the expression of Bub1 mRNA and protein. The tumors included PAs, warthin tumors MECs, ACCs and acinic cell carcinomas. Normal submandibular glands were used as control using real–time
quantitative reverse transcription-polymerase chain reaction (RT-PCR) or Western blotting. The mean expression levels of Bub1 mRNA and protein were higher in malignant tumors than normal submandibular glands and benign tumors. A significant association occurs between the level of Bub1 mRNA/protein expression and clinical stage in malignant tumors. Shigeishi et al. (2006) also analyzed Bub1 mRNA/protein expression and its relation with the proliferative activity monitored by the Ki-67 labeling index. Bub mRNA/protein expression and its relationship to the expression of proliferating cell nuclear antigen (PCNA) were also investigated by Western blotting. A significant correlation was found between Bub1 mRNA/protein expression and the Ki-67 labeling index in salivary gland tumors. Thus, increased expression of the human Bub1 gene appears to be linked to abnormal cell proliferation in malignant conditions.\(^{43}\)

The CIP/KIP family proteins, p21(WAF1/CIP1) and p27 (KIP1), occupy key positions in cell cycle regulation leading to an arrest of cell proliferation. They enable the DNA damage repair process. In several human tumors, a loss of these proteins is associated with poor clinical outcome. Affolter et al. (2005)\(^ {44}\) reported an altered expression of cell cycle regulators p21, p27 and p53 in tumors of salivary glands and paranasal sinuses. A comparison of the expression of p21, p27 and p53 in benign and malignant tumors of salivary glands and paranasal sinuses revealed that 78% of all ACCs had a complete loss of p27 expression whereas 60% of the adenocarcinomas overexpressed p27. The majority of cylindrical cell carcinomas showed distinct cytoplasmic accumulation of p27. All malignant tumors were positive for p21 after performing tumor specific antigen-immunohistochemistry, although 72% of those samples had shown weak to negative protein levels in conventional immunostaining.
Human CENP-F mRNA is closely linked to the increased or abnormal cell proliferation in salivary gland carcinomas.\textsuperscript{45} Proliferation associated antigen Ki-67 index is increased in CXPA, especially with adenocarcinoma, compared with PA and sialadenitis.\textsuperscript{44} High cyclin A expression is a useful marker for the pathological diagnosis of CXPA. Cyclin A is not expressed in normal salivary tissues of PA and CXPA.\textsuperscript{46}
VI. EIF4E, other Transcription Factors, and Apoptotic Factors in Malignant Transformation

Malignant transformation may result from selective dysregulation of cellular metabolism and growth. Tumorigenesis may result from alterations in many potential sites such as critical control points in the cell cycle, DNA replication and protein synthesis. There are many unanswered questions regarding the malignant transformation of a complex tumor arising from the salivary glands. For example, the longest report in the literature for malignant degeneration of a benign mixed tumor is 60 years from the initial diagnosis of the benign tumor. What keeps the tumor in check? What steps are necessary for the benign tumor to undergo malignant degeneration?

Eukaryotic initiation factor 4E (eIF4E) is an essential component of protein synthesis. It recognizes the 7-methylguanosine-containing cap at the 5’terminus of mRNA and assists in the transfer of the mRNA to the 48s ribosomal complex. eIF4E is a rate-limiting component of protein synthesis. mRNAs with long 5’ untranslated regions (5’UTRs) and complex secondary structures are more dependent on the presence of eIF4E because eIF4E facilitates the binding of the RNA helicase complex, eIF4F, which results in the unwinding of the 5’UTRs. The overabundance of eIF4E allows for the selective overexpression of translationally repressed mRNAs with long 5’UTRs. Specific proteins such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2) and cyclin D1 are up-regulated. The upregulation of the growth factors may alter cell cycle control, promote angiogenesis and lead to tumor growth. The
overexpression of eIF4E has been detected in other solid tumors including breast carcinoma. Significantly, eIF4E is not overexpressed in the benign counterpart of the corresponding tissue from non-cancer patients.

The initial work on eIF4E in human malignancies was performed in patients with breast carcinoma. In 112 breast carcinoma specimens examined, eIF4E overexpression was present at a range of 3-30 fold in all samples. In contrast, no eIF4E overexpression was detected in benign breast tissue. Furthermore, the degree of eIF4E overexpression appeared to have prognostic significance. In patients with Stage I, II or III breast carcinoma, the group with high eIF4E overexpression, experienced a statistically significant worse clinical outcome, with a higher risk for cancer recurrence and cancer-related death.

Clinically, in head and neck squamous cell carcinoma (HNCA), the presence of eIF4E elevation at the tumor free resection margin has a statistically significant increased risk for local recurrence. In 26 primary HNCAs, of the 12 with eIF4E overexpression at the surgical margin, five had recurrence at one year follow-up. In contrast, none of the patients with normal eIF4E displayed recurrence. In a study of 18 HNCA specimens, eight benign tumors (all PAs) and 10 benign specimens from non-cancer patients, there was progressive eIF4E gene amplification and overexpression when benign tissues from noncancer patients were compared with benign tumors from patients with PA and invasive carcinomas from patients with HNCA. Comparison of benign tumor and invasive carcinoma appears to be associated with increasing degree of eIF-4E gene amplification. Investigators suggest that molecular events, such as eIF4E gene amplification and overexpression, may precede cellular morphologic changes. A surgical
margin, which appears “tumor free” microscopically, may have elevated eIF4E protein levels and thus portends early recurrence. The ability to identify eIF4E gene amplification and/or overexpression may allow for potential intervention at the molecular level to reverse the dysregulation of the multiple critical molecules downstream and thus halt tumor progression.\textsuperscript{53}

High mobility group A2 (HMGA2) protein is a non-histone architectural transcription factor. HMGA2 is exclusively expressed in the nucleus of embryonic, but not of terminally differentiated cells and the aberrant expression of HMGA2 is associated with various benign tumors, including pleomorphic salivary adenoma. Lin et al.\textsuperscript{54} analyzed the expression pattern of HMGA2 by microarray to profile HMGA2-dependent salivary gene regulation. Using RT-PCR assays, the expression of a cluster of genes involved in cytokine signaling was verified to be upregulated in the salivary glands of AQP-5/HMGA2 mice. The genes included Il7r, Il2rg and Ptprc. In concert, the expression of a cluster of genes, namely Ppara, Phyh and Cidea, governing fatty acid and lipid metabolism was down-regulated by the HMGA2. Squamous carcinoma-like salivary tumors were observed in the transgenic mice but only at a low incidence. The AQP-5 promoter/enhancer–containing regions are sufficient to target salivary-specific transgene expression and HMGA2 may play a novel role in salivary epithelial cells.\textsuperscript{54}

Genome-wide, high–resolution array-comparative genomic hybridization (CGH) and fluorescence in situ hybridization were used to identify genes amplified in double minute chromosomes and homogeneously staining regions in PA and CXPA, and to identify additional genomic imbalances characteristic of these tumor types. Ten of 16 tumors analyzed showed amplification/gain of a 30-kb minimal common region,
consisting of the 5’–part of HMGA2 (encoding the three DNA-binding domains). Coamplification of MDM2 was found in nine tumors. Five tumors had cryptic HMGA2-WIF1 gene fusions with amplification of the fusion oncogene in four tumors. Expression analysis of eight amplified candidate genes in 12q revealed that tumors with amplification/rearrangement of HMGA2 and MDM2 had significantly higher expression levels when compared with tumors without amplification. Analysis of individual HMGA2 exons showed that the expression of exons 3-5 were substantially reduced when compared with exons 1-2 in 9 of 10 tumors with HMGA2 activation, indicating that gene fusions and rearrangements of HMGA2 are common in tumors with amplification. In addition, recurrent amplification/gains of 1q11-q32.1, 2p16.1-p12, 8q12.1, 8q22-24.1 and losses of 1p21.3-p21.1, 5q23.2-q31.2, 8p, 10q21.3 and 15q11.2 were identified. Collectively, HMGA2 and MDM2 were identified as amplification targets in PA and CXPA. Amplification of 12q genes (in particular MDM2), deletions of 5q23.2-q31.2, gains of 8q12.1(PLAG1) and 8q22.1-q24.1 (MYC) and amplification of ERBB2 may be important for malignant transformation of benign pleomorphic adenoma.

The signal transducer and activator of transcription (STAT3) is constitutively active in different types of malignant tumors. De Aruja et al.(2008) studied 50 biopsies of salivary gland tumors (9 PAs, 12 ACCs, 7 EMCs, 10 PLGAs and 12 MECs). In normal salivary gland, STAT3 was expressed in the cytoplasm and STAT3P in the nuclei of all tissue cells, except in large mucous acinar cells. In PA, the expression was the same as in normal glands. In malignant tumors, there were variations in the expression for STAT3 and STAT3P. The presence of STAT3 in the nuclei of the malignant tumor cells was significant, and most evident in the cribriform-type of adenoid cystic carcinoma. Both
loss and variation of STAT3P expression were also observed. The presence of STAT3 in
the nuclei of malignant salivary gland tumors may represent an important event in
oncogenesis probably contributing to tumor cell proliferation while blocking apoptosis.

X-linked inhibitor of apoptosis protein (XIAP) is a member of the inhibitor of
apoptosis proteins family of caspase inhibitors. Expression of XIAP in various neoplasms
has been associated with aggressive behavior. An immunohistochemical study of XIAP
expression in PA and CXPA adenoma revealed that tumor cells with strong staining
often exhibited cytological atypia in the form of nuclear enlargement and contour
irregularity, prominent nucleoli and eosinophilic cytoplasm. Mitotic activity was
occasionally seen in cellular areas expressing XIAP. All cases of CXPA demonstrated
strong staining in the carcinomatous component and weak staining in cellular areas of the
underlying PA. The results suggests an adenoma to adenocarcinoma model of
progression. Targeted therapy of XIAP may play a future role in the management of
CXPA.
VII. The Role of Oncogenes (*Plag-1, MDM2, Proto-oncogene bcl-2*) and Tumor Suppressor Genes (*p53, p21, hBD-1, p63*) in Tumor Progression

The development and progression of cancer are thought to be regulated by the expression of various oncogenes and tumor suppressor genes and this has been demonstrated for salivary gland tumors.\textsuperscript{58-61} As discussed earlier (Fig.4), *Plag1*, a novel developmentally regulated C2H2 zinc finger gene, is consistently rearranged and overexpressed in PAs of the salivary glands with 8q12 translocations. In transient transactivation assays, PLAG1 specifically activates transcription from its consensus DNA binding site, indicating that PLAG1 is a genuine transcription factor. Potential PLAG1 binding sites were found in the promoter 3 of the human insulin-like growth factor II (IGF-II) gene. PLAG1 binds IGF-II promoter 3 and stimulates its activity. Moreover, IGF-II transcripts derived from the P3 promoter are highly expressed in salivary gland adenomas overexpressing PLAG1. They are not detectable in NSG tissue and in adenomas without abnormal PLAG1 expression. PLAG1 and IGF-II expression display a strong correlation indicating that IGF-II is one of the PLAG1 target genes.\textsuperscript{62}

Other oncogenes, including ras, expression of p21 antigen, myc and c-erbB-2, have been identified in human salivary gland tumors.\textsuperscript{63-65} In one series of 476 tumors (29 PAs and 18 malignant lesions), up to 50% of the tumor cells stained positively for p21 antigen and 70% of the PAs were positive. In contrast, p21 expression was lower in malignant tumors, with fewer than 25% positive cells in the 66% that expressed this antigen. No p21 antigen was detectable in NSG by the immunohistochemical and immunoblotting
procedures used. A cultured cell line established from a human salivary gland adenocarcinoma also expressed elevated levels of ras mRNA and p21 antigen. Using immunohistochemistry and immunoblotting, rates of detection of the p185 protein product of the c-erbB-2 oncogene in salivary gland tumors are variable. To date, no correlation exists between oncogene expression and SGT subtype. Although ras expression in SGTs correlates with rearrangements of chromosome 8 (a chromosome with aberrations in human SGTs), the oncogene c-erbB-2 localizes to chromosome 17, abnormalities of which are not frequent in SGTs.

Genetic alterations of oncogene MDM2 promote malignant transformation of several human tumors. Schlott et al. (2001) used fluorescence-based PCR techniques and immunochemistry to analyze MDM2 gene amplification, gene expression, gene mutation, RNA splicing and accumulation. All 18 samples of benign and malignant tumors of the salivary gland contained non-amplified MDM2 genes with nonmutant zinc finger regions. However, in two benign and two malignant samples, novel MDM2 mRNA splicing variant types 1 and 2 were detected. Furthermore, three malignant tumors revealed significant nuclear MDM2 accumulation. This suggests that MDM2 gene mutation and gene amplification do not contribute to MDM2 accumulation detected in malignant tumors of the salivary gland. The role of novel MDM2 splicing variants in MDM2 expression and malignant transformation needs further study.

Although intraductal carcinoma has been demonstrated in intracapsular CXPA, the morphological and genetic stages of transformation of PA to CXPA are not fully understood. Ihrler et al. (2007) used immunohistochemical double-staining to detect p53 protein and cellular proliferation in different types of cells combined with mutational
analysis of the p53 gene in laser-microdissected material, intraductal carcinoma with high-grade cellular atypia, and p53 accumulated in 15 out of 19 cases. This frequent demonstration of intraductal carcinoma strongly indicates that a preinvasive lesion is likely to be a constant feature in the malignant transformation of PA to CXPA. Furthermore, p53 is a feature of CXPA developing from both primary and recurrent PA. The combined immunohistochemical and genetic data indicate that dysfunctional p53 is an early event in malignant transformation.

An immortalized duct/basal cell line (MSE) from the submandibular glands of p53-deficient mice was established by Obara et al. (2006).69 A variety of culture assays and xenograft experiments were conducted and cellular characteristics were analyzed using histological, immunohistochemical, ultrastructural and molecular techniques. Inoculation of a mixture of MSE and Matrigel reconstructed polarized ducts, whereas cotransplantation of MSE with both Matrigel and NIH3T3 (3T3) cells developed mixed tumors of adenoma and sarcoma. A daughter adenoma line (MSA) showed some transformed phenotype in vitro, but was marginally tumorigenic in vivo. PLAG1 was expressed in MSA but not in MSE. The intrinsic tumorigenic programs of p53 null salivary epithelium are promoted by 3T3 sarcoma-derived IGF-II in a paracrine manner through overexpression of PLAG1 and IGF-IR.

To illustrate the role of p63 and its truncated variants in SGTs, Foschini et al. (2005)70 studied 23 random SGTs and six NSGs immunohistochemically with anti-p63 antibody and by RT and nested PCR to detect p63 isoform expression. The NSGs displayed positive p63 antibody staining of basal and myoepithelial cells. The two main isoforms were present, whereas DeltaNp73L was absent. All tumors expressed p63
irrespective of their morphologic differentiation. The DeltaNp73L isoform was present in tumor tissue but absent in NSG tissue. Thus, p63, particularly its splice variant DeltaNp73L, appears to be involved in the neoplastic transformation of salivary glands.

In a similar study of p63 isotypes, Maruya et al. (2005) performed RT-PCR analysis of the markers for the TA and DeltaN isoforms of p63. Both p63 isoforms were either negative or weakly expressed in normal salivary gland tissues. TAp63 was highly expressed in most benign tumors and was either negative or weakly positive in most carcinomas. Conversely, DeltaNp63 staining was negative or faintly positive in most benign neoplasms, but highly expressed in ACC, MEC and myoepithelial carcinomas. Immunohistochemical analysis using anti-full-length p63 protein showed ubiquitous nuclear staining in basal and myoepithelial cells in both benign and malignant neoplasms. In addition, there was no correlation of the p63 expression with the expression of Notch ligand JAG1 gene. Jag1 was expressed in most benign and malignant tumors. Table 2 summarizes the p63 isotypes found in normal salivary glands and malignant tumors.
Table 2. p63 Isotypes in Normal Salivary Glands and Malignant Tumors  
(Maruya 2005)

<table>
<thead>
<tr>
<th></th>
<th>NSG</th>
<th>Malignant Tumors</th>
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<tbody>
<tr>
<td>Full length p63</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P63 isoform</td>
<td>Negative to weak</td>
<td></td>
</tr>
<tr>
<td>TAp63</td>
<td>+</td>
<td>Negative to weak</td>
</tr>
<tr>
<td>ΔNp63</td>
<td>Negative to weak</td>
<td>Highly expressed</td>
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</table>

WT-1 is a tumor suppressor gene that could have a role in malignant degeneration. Upregulation of WT-1 mRNA was observed in tumors of epithelial/myoepithelial phenotype in a study of 44 benign and malignant salivary gland tumors using gene expression analysis by RT-PCR. In the same study, FHIT (Fragile histidine triad gene) mRNA splicing did not appear to be involved in the genesis of salivary gland neoplasms. The tumor rejection genes, BAGE, GAGE-1/2, MAGE-1, MAGE-3 and HAGE, were more frequently, but not exclusively, expressed in malignant salivary gland tumors than in benign neoplasms.
VIII. Receptors Involved in Tumor Formation

The following receptors have been implicated in tumor formation: growth-factor binding receptors (HER-2), epidermal growth factor receptor (EGFR), transforming growth factor-alpha (TGFα), androgen receptor (AR), Her-2/Neu, platelet-derived growth factor (PDGF) A and PDGF alpha receptor, FGF-1 and FGF 2 receptors.

EGFR and TGF-α are significantly increased in CXPA as detected by immunohistochemical analysis. CXPA of the lacrimal gland displays a positive immunoreactivity for androgen receptor and BRST-2 supporting the hypothesis that the tumor is equivalent to a salivary duct carcinoma. Maatabayashi and Yoshihara (2007) examined the proliferative activity of 10 salivary gland CXPAs using immunohistochemistry. The control group consisted of 10 cases of other malignant tumors like adenocarcinomas (ACN), salivary duct carcinomas (SDC) and ten cases of PAs. The malignant component of CXPA showed a higher incidence of PCNA and Ki67 than the benign component of CXPA. Significant differences between the benign component of CXPA and PA were observed. Significantly higher p53, c-erB-2, EGFR overexpression was observed in the malignant component of CXPA compared to the benign component of CXPA. This suggests that CXPA acquired malignant characteristics while PA undergoes malignant transformation. Table 3 and Table 4 summarize the changes in receptor levels, p-53 and c-erB-2 expression in benign and malignant SGTs.
Table 3. Receptors Involved in Tumor Formation
(Katori et al, 2007; Takahira et al, 2007; Maatsubayashi and Yoshihara, 2007)

<table>
<thead>
<tr>
<th></th>
<th>Benign SGT PA</th>
<th>Malignant CXPA</th>
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<tbody>
<tr>
<td>EGFR</td>
<td>low</td>
<td>Increased (Katori)</td>
</tr>
<tr>
<td>TGF-α</td>
<td></td>
<td>Increased (Katori)</td>
</tr>
<tr>
<td>AR</td>
<td>+ lacrimal (Takahira)</td>
<td></td>
</tr>
<tr>
<td>BRST-2</td>
<td>+ lacrimal (Takahira)</td>
<td></td>
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</table>

Table 4. p53, E-erB-2, EGFR expression in CXPA and benign component of CXPA
(Maatsubayashi and Yoshihara 2007)

<table>
<thead>
<tr>
<th></th>
<th>Benign component of CXPA</th>
<th>CXPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>low</td>
<td>increased</td>
</tr>
<tr>
<td>E-erB-2</td>
<td>low</td>
<td>increased</td>
</tr>
<tr>
<td>EGFR</td>
<td>low</td>
<td>overexpression</td>
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</table>

Although the loss of p53 leads to malignant transformation and has been used as diagnostic markers for malignancy, DeRoche et al. (2008) showed p53 expression in benign PA. They used immunohistochemical stains for p53, AR and HER-2/neu in 41 histologically and clinically benign PAs. Therefore, these markers cannot be used to reliably predict early carcinomatous transformation in PA. In contrast to this study, Yamamoto et al. (1998) analyzed p53 abnormalities in eight patients with carcinoma in pleomorphic adenoma (CPA) by PCR-based assays and immunohistochemistry. Normal
salivary gland tissue, adenomatous, transitional adenocarcinomatous areas were microdissected from archival microslides and analyzed for allelic deletions of the p53 gene using two microsatellite markers at the p53 locus [the dinucleotide (CA)n repeat and pentanucleotide (AAAAT)n repeat]. LOH of the p53 gene was detected in 57% of adenomas, 86% of transitional lesions and 86% of carcinomas. In contrast, overexpression of p53 oncoprotein was noted immunohistochemically in 13% of adenomas, 50% of transitional areas and 75% of carcinomas. All of the tumors with immunoreactivity for p53 oncoprotein demonstrated LOH. Moreover, when LOH was present in adenomatous or transitional areas, the identical LOH was always detected in the corresponding carcinomatous areas in the same CPA tumors. These findings indicate that p53 gene mutation is an early event and occurs frequently at an early stage of precancerous lesions and may be responsible for most cases of malignant transformation of PA. However, since only eight cases were studied, this hypothesis should be validated by additional studies.

Deguchi et al. (1993) studied c-myc, ras p21 and p53 expression, and demonstrated a statistically significant increase in ras p21 and p53 expression between PA and its malignant form in human salivary glands. Immunoblotting assays clearly demonstrated the expression of c-myc and p53 gene products in both the benign and malignant forms of the pleomorphic adenoma and ras p21 in the malignant form. These results indicate that activation of c-myc and ras p21 proto-oncogenes and the involvement of p53 mutation may play important roles in the malignant transformation of salivary gland PA.
Growth signaling pathways undergo continuous activation in human tumors, commonly as a consequence of the overexpression of ligands and receptors such as platelet-derived growth factor (PDGF) and platelet-derived growth factor receptor (PDGFR). Hydrogen peroxide, produced after PDGFR activation, is essential for the sequential phosphorylation cascade that drives cell proliferation and migration. Peroxiredoxins are involved in growth factor signaling regulation and in the oxidative stress response by their ability to degrade hydrogen peroxide. Demasi et al. reported that the peroxiredoxin I, PDGF-A and PDGFR-α proteins were present in remnant PA to only a small extent. However, collectively, they were highly expressed as soon as the malignant phenotype was achieved and remained at elevated levels during progression to the advanced stages of CXPA. Their locations overlapped significantly, strengthening their connection to this growth-signaling pathway. The results indicate that CXPA cells are resistant to oxidative stress-induced apoptosis, conferred by high peroxiredoxin I concentrations and are characterized by sustained growth, reflecting PDGF-A and PDGFR-α overexpression.

FGF-1 and FGF-2 are heparin-binding polypeptides that express potent mitogenic properties in neoplastic cells. Myoken et al. (1996) studied the contribution of endogenous FGF-1 and FGF-2 to the autocrine growth of HSY human salivary gland adenocarcinoma cells in vitro. Both FGF-1 and FGF-2 were strongly expressed in the cytoplasm and in the nucleus in studies using specific monoclonal antibodies against them. HSY cells expressed the mRNA for FGF receptor-1 (FGFR-1) by RT-PCR, confirming the presence of high affinity FGF binding sites. There is no similar study to date on PA and CXPA.
Pleomorphic adenoma of the salivary glands does not appear to be a BRCA-1-dependent tumor in a Polish cohort as reported by Lubinski et al.\textsuperscript{81} Two hundred and sixty eight patients with mixed tumors of the salivary glands were examined for occurrence of three BRCA-1 mutations dominating in Poland. BRCA-1 mutation was detected in only one of the patients, a female affected by breast cancer and PA of the parotid gland. The parotid gland tumor showed clinical and histopathological features of typical pleomorphic adenoma with no morphological features of high-grade malignancy, which are characteristic of BRCA-1-dependent tumors.

Amplification of Her-2/neu oncogene and overexpression of its gene product have both prognostic and therapeutic implications in breast cancer. HER-2/neu is usually overexpressed in salivary duct carcinomas and in some cases of CXPA.\textsuperscript{82} Table 5 summarizes the changes in receptor levels.


<table>
<thead>
<tr>
<th>Receptor</th>
<th>Benign PA</th>
<th>Malignant CXPA</th>
<th>Malignant adenocarcinoma (in vitro)</th>
</tr>
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<tbody>
<tr>
<td>PDGF-A</td>
<td>Low (Demasi)</td>
<td>elevated</td>
<td></td>
</tr>
<tr>
<td>PDGFR-α</td>
<td>Low (Demasi)</td>
<td>elevated</td>
<td></td>
</tr>
<tr>
<td>FGF-1</td>
<td>Low (Demasi)</td>
<td>elevated</td>
<td>Cytoplasm, nucleus (Myoken)</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Low (Demasi)</td>
<td>elevated</td>
<td>Cytoplasm, nucleus (Myoken)</td>
</tr>
<tr>
<td>BRCA-1</td>
<td>Negative (Lubinski)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Her-2/neu</td>
<td>Overexpressed (Skalova)</td>
<td></td>
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</table>
IX. Other Signaling Pathways and Proteins Involved in Malignant Transformation

PLAG1 is involved in various human neoplasias, including pleomorphic adenomas of the salivary glands. The oncogenic role of PLAG1 was clearly demonstrated in two independent PLAG1 transgenic mouse founders, in which PLAG1 expression could be targeted to different tissues using the Cre/loxP system. MMTV-Cre-mediated targeted overexpression of PLAG1 in the salivary glands of double transgenic offspring mice, referred to as P1-MCre and P2-MCre mice, induced PA in this organ. IGF2, a genuine PLAG1 target gene, was highly upregulated in those tumors as well as in human PAs of the salivary glands.  

Declercq et al. (2008) provided further evidence for the role of IGF2 in PLAG1-induced tumorigenesis. Inactivation of IGF2 in P1-MCre mice leads to a significant delay in tumor development. Since tumor development is not fully abrogated by inactivation of IGF2, other signaling pathways are likely to contribute to PLAG1-induced tumorigenesis as well. Additional studies revealed that several genes such as H19, Dlk1, Gtl2, IGFbp2, IGFbp3 and genes involved in Wnt signaling such as Wnt6, Cyclin D1 and beta-catenin are upregulated in P1-MCre mice in which IGF2 is inactivated. Thus, several genes associated with IGF and Wnt signaling are upregulated in PLAG1-induced PAs. Inactivation of IGF2 does not affect upregulation of genes associated with Wnt signaling, which suggests that both signaling pathways are involved.
The cadherin/catenin cell-cell adhesion complex is another key signaling pathway. Changes in beta-catenin distribution has been associated with several human cancers including salivary gland tumors. Genelhu et al. (2007) studied the immunolocalization of beta-catenin in a series of PA and CXPA using immunohistochemistry with a monoclonal E-5 antibody against beta-catenin. Cell membrane/cytoplasmic staining of beta-catenin was observed in normal gland parenchyma, PA, and in well-differentiated CXPA. Cytoplasmic/nuclear beta-catenin staining was observed in poorly differentiated carcinomas and in one case of PA. Genelhu’s data showed decreased cell membrane beta-catenin expression in higher-grade tumors suggesting that beta-catenin may play an important role in histologic differentiation and transition to the malignant phenotype of CXPA. The transformation from PA to malignancy may involve additional changes or lack of regulation in these signaling pathways.

Defensins such as hBD-1,-2,-3 are positively charged peptides with molecular weights ranging from 3.5 to 6.5 kDa. They have antimicrobial potency because of their ability to bind to glycoproteins and destabilize membranes. Defensins have been detected in a variety of diverse tissues including the epithelia of the oral cavity, the gastrointestinal and respiratory tract, the urinary tract and the vagina as well as in salivary glands. A cancer specific loss of hBD-1 occurs in 90% of renal clear cell carcinoma cases and 82% of malignant prostate cancer cases. Wenghoefer et al. (2008) examined 21 tissue samples of seven benign SGTs, seven malignant SGTs and seven healthy salivary glands. The constitutively expressed hBD-1 shifted from the cytoplasm to the nucleus of the tumor cells in malignant SGTs. Thus, hBD-1 may function as a tumor suppressor.
gene and play a key role in the malignant progression of epithelial tumors. Further, the PAs showed both cytoplasmic and weak nuclear hBD-1 staining, supporting the hypothesis on the role of nuclear hBD-1 translocation in the ability of these tumors to recur or undergo a malignant transformation.\textsuperscript{86}

Tenascin is a matricellular protein that has been studied in several tumor types. Its expression has been correlated with tumor morphogenesis as well as with local invasiveness and tumor metastatic behavior. The distribution pattern of tenascin in a series of 63 PAs and 20 CXPAs was studied immunohistochemically.\textsuperscript{87} Ten normal adult salivary glands were used as controls. Tenascin surrounded the excretory ducts of normal adult salivary gland tissue but was absent in the basement membrane compartment of both benign and malignant mixed tumors. These findings strongly support the hypothesis that tenascin deposition is involved in the mechanisms of malignant transformation of PAs into carcinomas as well as being associated with clinical disease progression.

Galectins are a family of non-integrin beta-galactosidase-binding lectins. Altered expression of galectins has been associated with neoplastic transformation and progression in several human tumors. Immunohistochemical galectin-3 expression was limited to ductal cells, but galectin-1 was usually faintly detected in ductal cells and strongly positive in myoepithelial cells. In benign tumors, galectin-3 maintained the ductal localization, but galectin-1 showed variable expression in ductal and myoepithelial cells. In malignant tumors, most of the polymorphous low-grade adenocarcinomas and CXPAs expressed both galectins, whereas ACCs and acinic cell carcinomas showed dramatically reduced galectin-3 expression and heterogeneous galectin-1 staining.
Galectin-3, and to a lesser extent galectin-1, appear to have altered localization and expression in salivary gland carcinomas. 88
Angiogenesis and Its Role in Malignant Transformation

Angiogenesis is an important component in the progression of a benign tumor to malignancy. Soares et al.\textsuperscript{89} assessed tumor vascularization in histological samples of early CXPA and PA by measuring total microvascular area (TVA) and microvessel density (MVD) using CD34 and CD105 antibodies. MVD for CD105 increased significantly during tumor progression, but not CD34 MVD. Comparing widely invasive CXPA with and without myoepithelial differentiation, CXPA with myoepithelial differentiation showed a significantly lower number of CD105 positive vessels but revealed higher TVA values. In these tumors, the neoplastic cells usually formed larger hypovascularized aggregates that were often surrounded by large-sized vessels. Localization of the antibody CD105 revealed an angiogenic switch during the progression from adenoma to carcinoma in salivary glands. The degree of angiogenesis and the TVA have distinctive patterns in CXPA with and without myoepithelial differentiation. Low angiogenesis associated with high TVA value is more characteristic of CXPA with myoepithelial differentiation.\textsuperscript{89}
XI. External Factors and their Effects on Proto-oncogenes

External factors and their effects on proto-oncogenes have been studied in salivary gland tumors. The proto-oncogenes c-fos and c-jun express proteins targeted into the nucleus. The fos and jun proteins form a heterodimeric complex that binds to regulatory elements in the promoter region of specific genes to influence their transcription. Thus, the fos and jun proteins appear to link extracellular stimuli to short- and long-term functional changes in cells. Observations by Kousvelari et al. (1990) suggested that beta-adrenergic receptor stimulation of rat parotid acinar cells in vitro or addition of 8-BrcAMP in a rat submandibular cell line (RSMT-A5) increased the expression of the c-fos gene in a time-dependent manner. In another experiment by the same group, c-jun expression in both salivary cell types had a similar pattern of expression for this proto-oncogene. Conversely, treatment of rats with isoproterenol for nine days resulted in the appearance of two c-abl mRNAs of unique size, in addition to the known 5.3-kb c-abl transcripts. Thus, beta-adrenergic receptor stimulation or exposure to 8-BrcAMP may induce the early expression of the nuclear proto-oncogenes c-fos and c-jun before changes are noted in salivary epithelial cell proliferation.

To what extent does the external environment (breakage of the capsule in surgical operations) contribute to malignant transformation? What factors in the tumor microenvironment cause the benign tumor to degenerate into malignancy? There are as yet no known factors proven directly to cause malignant transformation in PA.
XII. Possible Mechanisms of Malignant Transformation of PA to CXPA in Salivary Glands

The classic model for malignant transformation is colon cancer. LOH on the long arm of Chromosome 5 (5q), the site of the Adenomatous Polyposis Coli (APC) gene is the first step in the change of normal colon epithelium to an early-stage adenoma. Larger adenomas tend to have high rates of LOH on the long arm of Chromosome 18 (18q) and short arm of Chromosome 17 (17p). Figure 5 is the classical “Vogelgram” developed by Fearon and Vogelstein, demonstrating the multi-step progression of the germ-line APC mutation to familial adenopolyposis and to fullblown colorectal cancer.

Figure 5. Colon tumor progression and loss of heterozygosity in various chromosomal arms (Fearon and Vogelstein, 1990)

The tumor suppressor genes involved are APC on Chromosome 5q and p53 on Chromosome 17p. The oncogene K-ras is involved in the adenomatous phase of the tumor while 17p and 8p loss trigger the final steps in the malignant degeneration. More
recently, a loss of tumor suppressor gene DCC on chromosome 18 has been identified updating the model for multistage tumorigenesis in colon cancer.\textsuperscript{93}

Although there is a paucity of research data on the malignant transformation of PA, possible mechanisms for transformation may be hypothesized based on current findings on PAs and malignant neoplasms of the salivary gland. In an existing PA, PLAG-1 is upregulated which then upregulates IGF-II, which in turn signals a mitogenic pathway. The tumor could stay in a balance for years as long as the apoptotic mechanisms are intact. Normal salivary glands are negative for cyclin D1, negative for CDK-4, positive for p16, but have significant increases in pRB and moderate expression of E2F-1. In contrast, in PA (not BRCA-1 dependent) cdk-4, p16, E2F-1 are increased but pRB is decreased. The known common pathways found in malignant transformation of PA of the salivary glands is the involvement of p53, c-myc and c-ras. A possible model for a normal salivary gland developing into pleomorphic adenoma is proposed below (Fig.6).

\textbf{Figure 6. A model for a normal salivary gland developing into pleomorphic adenoma.}
The first step from a normal salivary gland to a benign PA is presently hypothesized to be the activation of \textit{PLAG1}, a gene for a transcription factor on chromosome 8q12i. This is analogous to the \textit{APC} gene identified in the model of colon cancer. The benign PA may remain stationary for long periods of time, as long as a 60 year incubation period. Other PAs show a deletion on chromosome 3p21 or 3q21-27. The cell cycle gene CDC25A is found in this area. Abnormalities in chromosome 12 q13-15 also have also been found. The gene involved in this chromosome has not been identified. The changes cause an increase in cell mass but the apoptotic mechanisms may still be in place, thus, there is some control in the balance of cell proliferation and cell death. The steady-state is maintained at a higher population level and histologically, there are no signs of cellular atypia.

The activation of \textit{PLAG1} may not be sufficient to drive the adenoma into a full blown carcinoma. For the benign tumor to evolve into a carcinoma, several models can be proposed. Based on the literature reviewed, one such model is diagrammed on the next page (Fig.7).
In this model, a combination of tumor suppressor gene inactivation and oncogene activation is invoked. The inactivation of p-53, already found in some PA, could set the stage for further cell cycle dysregulation. C-ras could be involved because of the evidence that a deletion on chromosome 6 q22-25 is present in malignant salivary gland tumors. Several other oncogenes are located in this region namely: c-syn, c-myb and c-mas. However, there is no experimental data to support which of these oncogenes are involved in early malignant transformation.

p53 inactivation is confirmed by the loss of a segment of chromosome 17p13 in a large sample of malignant tumors. The DCC gene is implicated because of an abnormality in chromosome 18 q. Subsequent changes occur in p21. A combination of these genetic abnormalities may trigger the step toward carcinogenesis. The intracellular
signaling circuitry and collaboration between cancer-associated genes is illustrated in the next figure (Fig.8).

**Figure 8. The intracellular signaling circuitry and collaboration between cancer associated genes.** (Hanahan and Weinberg, 2000)

Different subcircuits are involved in regulating distinct cell physiologic processes. The circuits include: (1) growth-promoting, mitogenic circuit (2) circuit governing growth-inhibitory signals (3) circuit governing apoptosis (4) circuit governing invasiveness and metastasis.

Similar to the progression in chromosomal defects correlating with the progression into malignancy of colon tumors, PAs may degenerate into malignancy through the successive or simultaneous abnormalities of the tumor suppressor genes p53,
pRb, p27, p21 and oncogenes c-ras, c-myb and c-mas. Both the mitogenic circuitry and growth inhibition circuitry are affected.

A vast number of signaling pathways influence cell cycle progression. The decision to proceed with the cell cycle is made in G1. Increased p16 and CDK-4-cyclin in PA leads to activation of E2F which leads to cell proliferation. The oncogenes similarly increase cell proliferation by changing the levels in gene expression. With the mutation in p53 and p21, there is a decrease in pRb, resulting in cell proliferation. Several human cancers have been documented to have molecular changes in the cell cycle regulators.

In salivary gland tumors, there is a decrease in pRb as a result of increased levels of cyclin D1 and CDK4 leading to pRb phosphorylation. pRb phosphorylation leads to the activation of the E2F transcription factors. Deregulation of the pRb pathway is an integral characteristic of most, if not all, tumor cells. Interestingly, pRb appears to decrease even in benign PA. In PA, there is a correlation between cyclin D1 and E2F-1, cdK4 and E2F-1 as well as p16 and E2F-1. What tilts the balance to malignant transformation? To date, no evidence exists for a specific change coupled with the pRb phosphorylation that leads to malignancy. With the inactivation of Rb, the cell cycle progression in G1 becomes mitogen-independent. Mitogen signals promote Cyclin D1 and E expression, levels of which are increased in SGTs.

Other growth signaling pathways are affected in salivary gland tumors. In CXPA, there is EGF receptor overexpression. Receptor overexpression may enable the cancer cell to become hyperresponsive to ambient levels of growth factors that normally would not trigger proliferation. The nature of the EGF receptors in these tumors have not been
studied. There could be structural alteration of receptors that may cause the receptor to fire constitutively. Other receptors affected include AR and BRST-2 which also affect gene expression and cell proliferation.

As reported in the literature, c-ras, c-myb, c-syn and c-mas may be activated in malignant tumors of salivary glands. Downstream, there is activation of the Ras-Raf-MAPK cascade. Ras proteins, when present in structurally altered forms, enable the tumors to release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators. This cascade is also linked via a variety of cross-talking connections with other pathways. This is a rich area for future research.

The successive changes in IGF and Wnt signaling also cause cell proliferation. The presence of STAT3 in the nuclei of carcinoma cells leads to a block in the apoptotic pathway which could contribute to the increasing malignancy of the tumor. Other factors like DNA hypomethylation have not been investigated in SGTs. In summary, in malignant degeneration of pleomorphic adenoma there is:

1. Growth signal self-sufficiency through pRb phosphorylation
2. Insensitivity to growth-inhibitory signals
3. Evasion of programmed cell death (apoptosis) through XIAP
4. Sustained angiogenesis through VEGF and FGF.

Unlimited replicative ability in malignancies may be due to telomerase activity. This has not been demonstrated in any of the SGTs to date. Conflicting reports occur with regards to p27, a CKI inhibitor of the KIP class. In one report, a majority of ACCs of salivary glands have a complete loss of p27 expression while a majority of
adenocarcinomas overexpressed the protein. In this same investigation, all malignant tumors were positive for p21 expression using tumor-specific antigen immunostaining. These reports indicate that there may be stages in the malignant process where p21 and p27 levels are maintained. It is possible that a loss of both p27 and p21 could lead to a more aggressive tumor growth.

G1 progression is negatively regulated by cyclin-dependent kinase inhibitor proteins or CKIs. Mammalian CKIs belong to two classes, INK4 and KIP. INK4 proteins block cyclin D-CDK4/6 activity while KIP proteins block cyclin A-CDK-2 activity. The blocking of the cycE-CDK2 activity by p27 and p21 could cause unlimited replicative potential in SGTs.
XIII. Application of Previous Studies on Salivary Gland Tumors to the Study of Lacrimal Gland Tumors

Studies on lacrimal gland tumors may be done in parallel with salivary gland tumor studies. Cytogenetic investigation of benign and malignant lacrimal gland neoplasms revealed recurring chromosomal abnormalities involving chromosomes 3, 8, 9 and 12, features that are similar to those found in salivary gland tumors. Thus, common mechanisms may be involved in the neoplastic proliferation of these histologically related tumors.\textsuperscript{95} In another investigation examining head and neck carcinomas, the most common aberrations detected were deletions of the long arm of chromosome 6.\textsuperscript{96} That abnormality has been detected in all types of non-squamous cell carcinomas except CXPA. The samples were taken from short term cultures from 12 non-squamous cell carcinomas of the head and neck including acinic cell carcinomas, ACC, MECs, adenocarcinomas and carcinomas in pleomorphic adenoma. Clonal chromosome aberrations were detected in all but one adenocarcinoma. Two aberrations closely associated with tumor type were t(6:9) (q21-24;p13-23) that were observed in ACCs and structural rearrangements of 8q12-13 which were detected in PA.\textsuperscript{96}

The expression of p21ras in PA of the lacrimal gland was monitored using the monoclonal antibody F-132-62 and the nuclear DNA content in the tumor was assayed by image analysis. Positive correlation was observed between the expression of p21ras and DNA ploidy distribution pattern. Increased expression of p21ras in PA appears to be related to the promotion and progression of PA of the lacrimal gland.\textsuperscript{97}
The expression of the EGF receptor in pleomorphic tumors of the lacrimal gland is also related to proliferative activity of the tumor cells. DNA ploidy distribution pattern of the tumor with positive EGF receptor expression showed mainly two peaks. Using a polyclonal antibody to the c-erB-2 oncogene product (ErbB-2 protein), an immunohistochemical study on the expression of ErbB-2 protein in lacrimal gland tumors demonstrated expression of ErbB-2 protein in four of 12 cases of PA, but was not observed in two cases of ACC and in one normal lacrimal gland. The immunohistochemical reaction was localized in the cell membrane and cytoplasm and was more intense in the former. Cells in the epithelium of ductal cell lineage were strongly stained and some cells in the myxoid/chondroid areas were weakly stained. The correlation of ErbB-2 protein levels with malignant lacrimal gland tumors in general is not clear but PAs of the lacrimal gland could have some relation with this protein.

To date, the change in PLAG activity is not documented in benign mixed tumors of the lacrimal gland. This could be a starting point to determine if PLAG1 overexpression causes PA of the lacrimal glands. Additional research is needed to elucidate whether the rest of the oncogenes and tumor suppressor genes involved in malignancies of the salivary gland are also involved in malignant transformation of tumors in the lacrimal gland.
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Appendix:

Histology of Normal Lacrimal Glands (10X). (With permission from the Institute of Ophthalmology, University of the Philippines Manila)

Histology of Normal Salivary Glands and Benign Mixed tumor (10X). (With permission from the Department of Pathology, College of Medicine, University of the Philippines Manila)
Pleomorphic Adenoma of the Lacrimal Glands (10X) (With permission from the Institute of Ophthalmology, University of the Philippines Manila)

Malignant Mixed Tumor of the Lacrimal Glands (10X) (Institute of Ophthalmology, University of the Philippines Manila)