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SALIVARY GLAND CANCER IN FINLAND

Incidence, Histological Distribution, Outcome and Prognostic Factors

by

Heikki Luukkaa

ACADEMIC DISSERTATION

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TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2010

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This work is dedicated to all patients with cancer in the salivary glands with a hope of achieving better cure.

Heikki Luukkaa.

Salivary gland cancer in Finland: Incidence, histological distribution, outcome and prognostic factors.

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ABSTRACT

Salivary gland cancer (SGC) is a rare cancer. The histological classification of SGC is complex and its biological behavior highly variable: it may vary from a low-grade tumor to a high-grade and often fatal malignancy. These circumstances make this cancer a diagnostic and therapeutic challenge. Older age and exposure to ionizing radiation are known risk factors. The mainstay of treatment is surgery combined with adjuvant radiation therapy, when appropriate. In addition to the histological type, the only well known prognostic factor is the TNM classification, which describes the tumor size and the amount of metastases.

This study was performed using a full population-based nationwide cohort of SGC patients and tumors diagnosed in Finland in 1991-1996. The annual incidence of SGC in the entire population was, on average, 47.7 per year. By histological re-evaluation of 237 specimens the most frequent histological types were the adenoid cystic carcinoma (n=65; 27%), the mucoepidermoid carcinoma (n=45; 19%) and the acinic cell carcinoma (n=41; 17%). The highest 10-year disease-specific survival rate occurred among patients with acinic cell carcinoma (90%), followed by mucoepidermoid carcinoma (81%) and adenoid cystic carcinoma (60%).

A high volume-corrected index (VCI) of Ki-67 correlated with worse survival of patients with SGC. Computer-assisted morphometric analyses of CD34-positive vessels indicated an unfavorable prognosis for patients with mucoepidermoid carcinoma and an association with poor survival among patients with acinic cell carcinoma. A high level of expression of matrix metalloproteinase-9 (MMP-9) showed a trend for a poorer prognosis in salivary duct carcinoma, and a high level of MMP-13 and a low level of MMP-1 had a trend for a poorer prognosis of patients with SGC. A low level of MMP-7 was associated with a poor prognosis of patients with acinic cell and mucoepidermoid carcinoma.

Key words: Salivary gland neoplasms, epidemiology, MMPs, matrix metalloproteinases, Ki-67 antigen, tumor suppressor protein p53, computer-assisted image processing, CD34 antigen, genes HER-2, carcinoma acinar cell, carcinoma mucoepidermoid, carcinoma adenoid cystic, prognosis

Heikki Luukkaa.

Sylkirauhassyöpä Suomessa: Esiintyvyys, histologia, hoitotulokset ja ennusteeseen vaikuttavat tekijät.

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TIIVISTELMÄ

Sylkirauhassyöpä on harvinainen pään- ja kaulan alueella esiintyvä syöpätauti. Sen histologinen luokittelu on monimutkainen ja kasvaimien kliininen ilmentymä vaihtelee hitaasti etenevästä matala-asteisesti malignista kasvaimesta huonosti erilaistuneeseen korkea-asteisesti pahanlaatuiseen ja usein kohtalokkaaseen tautimuotoon. Tämän vuoksi sylkirauhassyöpä on sekä diagnostiikan että hoidon onnistumisen osalta erittäin haasteellisen syöpätauti. Potilaan korkea ikä ja altistuminen ionisoivalle säteilylle ovat tunnettuja etiologisia tekijöitä. Hoidon kulmakivi on kasvaimen kirurginen poisto täydennettynä tapauskohtaisella sädehoidolla. Histologisen tyyppin lisäksi ainoa tunnettu ennusteellinen tekijä on TNM-luokitus, joka kuvaa kasvaimen kokoa ja levinneisyyttä imusolmukkeisiin ja etäpesäkkeinä.

Käsillä oleva tutkimus perustui kansalliseen koko väestön kattavaan sylkirauhassyöpäpotilasaineistoon, joka kerättiin Suomesta vuosilta 1991–1996. Sylkirauhassyövän esiintyvyys oli keskimäärin 47,7 tapausta vuodessa. Histologinen uudelleenarviointi tehtiin 237 tapauksesta, jossa yleisimmät tautityypit olivat: adenokystinen karsinooma (n=65; 27 %), mukoepidermoidikarsinooma (n=45; 19 %) ja asinussolukarsinooma (n=41; 17 %). Kymmenen vuoden tautispesifinen elossaololuku oli asinussolukarsinoomassa 90 %, mukoepidermoidikarsinoomassa 81% ja adenokystisessä karsinoomassa 60%.

Korkea proliferaatioantigeeni Ki-67:n volyymikorjattu suhdeluku (VCI) korreloi sylkirauhassyövän huonoon ennusteeseen. Tietokoneavusteisissa morfometria-analyyseissä CD34-positiivisista suonista tehdyt havainnot korreloivat potilaan huonompaan ennusteeseen mukoepidermoidikarsinoomassa ja yhteys huonompaan ennusteeseen löydettiin myös asinussolukarsinoomassa. Matriksin metalloproteiinaasin (MMP)-9 korkeampi taso osoitti suuntaa salivary duct syövän huonompaan ennusteeseen ja MMP-13:n korkeampi taso sekä MMP-1:n matalampi taso osoittivat suuntaa sylkirauhassyövän huonompaan ennusteeseen. MMP-7:n matalampi taso oli yhteydessä asinussolukarsinooman ja mukoepidermoidikarsinooman huonompaan ennusteeseen.

Avainsanat: Sylkirauhasten kasvaimet, epidemiologia, matriksimetalloproteiinaasit, Ki-67-antigeeni, kasvainsalpaajaproteiini p53, tietokoneavusteinen kuvankäsittely, CD34 antigeenit, erbB-2, asinussolukarsinooma, mukoepidermoidikarsinooma, adenokystinen karsinooma, ennuste

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ABBREVIATIONS

AcCC	acinic cell carcinoma
AdCC	adenoid cystic carcinoma
Ca-ex-PA	carcinoma ex pleomorphic adenoma
CAQIA	computer-assisted quantitative image analysis
CD31	endothelial cell-specific antibody
CD34	endothelial cell-specific antibody
CD105	endothelial cell-specific antibody
CI	confidence interval
C-KIT	the transmembrane tyrosine kinase receptor (CD117)
CT	computed tomography
DEMC	dedifferentiated epithelial-myoeepithelial carcinoma
DNA	deoxyribonucleinic acid
EBV	Epstein-Barr virus
ECM	extracellular matrix
EGFR	epidermal growth factor receptor family
EIA	enzyme immunoassay
EMC	epithelial-myoeepithelial carcinoma
factor VIII	endothelial cell-specific antibody
Fas-ligand	cell surface molecules
FCR	Finnish Cancer Registry
FNAC	fine-needle aspiration cytology
Gy	gray
HNSCC	head and neck squamous cell carcinoma
HER-2/neu	human epidermal growth receptor-2 (c-ErbB-2)
HER-1	EGFR (epidermal growth factor receptor)
HR	hazard ratio
IMRT	intensity-modulated radiation therapy
Ki-67	nuclear antigen Ki-67
LCC	large cell carcinoma
MALT	mucosa-associated lymphoid tissue type lymphomas
MDM2	negative regulator of the p53 tumor suppressor
MEC	mucoepidermoid carcinoma
MIB-1	monoclonal antibody for Ki-67
<i>MKI67</i>	gene, which encodes the Ki-67 protein
MMP	matrix metalloproteinase
MMP-1	collagenase-1
MMP-7	matrilysin-1

MMP-9	gelatinase B
MMP-13	collagenase-3
MRI	magnetic resonance imaging
NCCN	National Comprehensive Cancer Network
O/E	observed-to-expected ratio
OEMC	oncocytic epithelial-myoeplithelial carcinoma
OR	odds ratio
p53	p53 tumor suppressor gene / p53 tumor suppressor protein
PLCC	polymorphous low-grade adenocarcinoma
pro- α -defensin	cell surface molecule
RECK	membrane-anchored inhibitor of matrix metalloproteinases
S-100	calcium binding protein
SCC	squamous cell carcinoma
SDC	salivary duct carcinoma
SEER	the Surveillance, Epidemiology, and End Results
SGC	salivary gland cancer
SISH	silver enhanced <i>in situ</i> hybridization
SmCC	small cell carcinoma
TIMP1	tissue metalloproteinase inhibitor 1
TNF- α	pro-tumor necrosis factor
TNM	cancer staging system
TP53	gene, for tumor suppressor protein p53
UV	ultraviolet
VCI	volume-corrected index
VMI	volume-corrected mitotic index
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

The study is based on the following publications, which are referred to in the text by the Roman numerals I-VII.

- I Luukkaa H, Klemi P, Leivo I, Koivunen P, Laranne J, Mäkitie A, Virtaniemi J, Hinkka S, Grénman R. Salivary gland cancer in Finland 1991-1996, an evaluation of 237 cases. *Acta Otolaryngol.* 2005;125:207-14.
- II Luukkaa H, Klemi P, Leivo I, Vahlberg T, Grénman R. Prognostic significance of Ki-67 and p53 as tumor markers in salivary gland malignancies in Finland, an evaluation of 212 cases. *Acta Oncol.* 2006;45:669-75.
- III Luukkaa H, Laitakari J, Vahlberg T, Klemi P, Grénman R. Morphometric analysis using automated image analysis of CD34-positive vessels in salivary gland acinic cell carcinoma. *Acta Otolaryngol.* 2007;127:869-73.
- IV Luukkaa H, Klemi P, Hirsimäki P, Vahlberg T, Kivisaari A, Kähäri V-M, Grénman R. Matrix metalloproteinase (MMP) -1, -9 and -13 as prognostic factors in salivary gland cancer. *Acta Otolaryngol.* 2008;128:482-90.
- V Luukkaa H, Klemi P, Hirsimäki P, Vahlberg T, Kivisaari A, Kähäri V-M, Grénman R. Matrix metalloproteinase (MMP) -7 in salivary gland cancer. *Acta Oncol.* 2010;49:85-90.
- VI Luukkaa H, Laitakari J, Vahlberg T, Klemi P, Grénman R. Morphometric analysis of CD34-positive vessels in salivary gland adenoid cystic and mucoepidermoid carcinomas. *J Oral Pathol Med.* 2009;38:695-700.
- VII Luukkaa H, Klemi P, Leivo I, Mäkitie A, Irish J, Gilbert R, Perez-Ordóñez B, Hirsimäki P, Vahlberg T, Kivisaari A, Kähäri V-M, Grénman R. Expression of matrix metalloproteinases (MMP) -1, -7, -9 -13, Ki-67 and HER-2 in epithelial-myoepithelial salivary gland cancer (EMC). *Head Neck.* 2010;32:1019-27.

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1. INTRODUCTION

Salivary gland cancer (SGC) is an uncommon disease of the upper aerodigestive tract. Tumors arising in these glands comprise only about 4 percent of all epithelial malignant neoplasms encountered in the head and neck; the age-adjusted incidence is 1.5 per 100 000 person-years (Finnish Cancer Registry 2009). The etiology, prognostic factors and risk factors are poorly defined. Considering the diversity of anatomic sites and the number of different histological subtypes of these tumors, it is easy to understand how otolaryngologists, head and neck surgeons, oncologists and pathologists accumulate only restricted exposure to specific types of salivary gland cancer. Many of these tumors behave in an indolent fashion and some histological types tend to recur late. Thus, there is a call for prolonged follow-up to improve the ability of the clinician to draw conclusions about the efficacy of treatment. Due to a lack of long-term follow-up, prevention, screening, registration and risk factors are poorly known (Spiro and Spiro 2001).

The morphological diversity of SGC poses a great challenge to diagnosis and classification of these tumors. They vary morphologically not only among individual tumors but there is also heterogeneity within the one and the same tumor mass in an individual patient (Eveson 1992). The first well recognized histological classification of SGC was published in 1953 (Foote and Frazel 1953). The World Health Organization (WHO) published a classification in 1991 (Seifert 1991) which has been updated several times, and the most recent version is from 2005, where 24 SGCs of epithelial origin are included (Barnes et al. 2005).

A diagnosis of salivary gland neoplasm must be considered in any patient who presents with a mass at the site of the parotid or submandibular glands or with a submucosal mass in the oral cavity or the pharynx. The mass may have been present for years. Major salivary gland tumors should not be surgically biopsied, since there is a risk of tumor spread and damage to the facial nerve. A preoperative sonography combined with fine needle aspiration cytology (FNAC) and in some cases CT scan and MRI, give often the necessary information prior to surgery, which is the standard management of salivary gland tumors. Postoperative radiotherapy is tailored individually, when indicated. The histological type, TNM-classification and stage determine how the tumor is to be treated definitively. However, the most aggressive cancer types necessitate more effective therapy than low-grade diseases (Witt 2004).

The most common genetic change in any human cancer is mutation of the p53 tumor suppressor gene which has been linked with tumor growth and progression (Levine et al. 1991, Vogelstein et al. 2000). The Ki-67 proliferation marker is a prognostic factor in numerous types of cancer, including some salivary gland carcinomas: the mucoepidermoid carcinoma (MEC) (Skalova et al. 1994a, Kiyoshima et al. 2001), acinic

cell carcinoma (AcCC) (Skalova et al. 1994b and Hellquist et al. 1997), adenoid cystic carcinoma (AdCC) (Nordgård et al. 1997) and carcinoma ex pleomorphic adenoma (Ca-ex-PA) (Xin and Paulino 2002).

High levels of matrix metalloproteinases (MMPs) have been associated with invasive properties of cancer and the role of the MMPs as prognostic factors has been studied widely in different cancer types. The MMPs are expressed in salivary gland cancer, but their role as prognostic factors is unclear (Azuma et al. 1993, Soini and Autio-Harminen 1993, Freitas et al. 2004, Kayano et al. 2004, Nagel et al. 2004, Westernoff et al. 2005).

The c-ErbB-2 gene, also known as HER2/neu, codes for the ErbB2/human epidermal growth factor receptor-2 (HER-2). HER-2 is overexpressed on the surface of some forms of ductal carcinoma cells of the breast and is associated with a poor prognosis. In salivary gland carcinomas, HER-2 is often expressed particularly in salivary duct carcinoma (Williams et al. 2007). Trastuzumab (Herceptin[®], Genentech Inc., San Francisco, CA, USA) is a monoclonal antibody against the HER-2-receptor used therapeutically. Patients with HER-2 positive breast cancer treated with trastuzumab have had longer disease-free survival and overall survival than HER-2 negative patients. Numerous studies are underway to develop more drugs directed towards specific molecular sites related to cell growth, cell differentiation or cell signaling. Thus far they have not received general acceptance for the treatment of SGC.

Increased angiogenesis is present in over 50 various disease states, as cancer, coronary heart disease, rheumatoid arthritis, psoriasis and diabetes mellitus (Folkman 2006a). Angiogenesis is essential for the growth, invasion and metastasis of a solid neoplasm. In epithelial cancer of different organs, angiogenesis has been associated with prognosis in more than one hundred studies (Makrilia et al. 2009). Moreover, angiogenesis has been proposed to have a prognostic role in head and neck cancer (Ascani et al. 2005, Erovcic et al. 2005). The vessels of tumors can be identified under the microscope with computer-assisted quantitative image analysis (CAQIA) (Laitakari et al. 2004) after staining with different endothelial cell-specific antibodies, *e.g.*, CD34 (Meert et al. 2002). CAQIA may be applied for combining morphological and functional features of a tissue (Baak and Janssen 2004). Comparisons of results within the same study are more reliable using computer-assisted morphometry than visual analysis or histoscore determinations (Barth et al. 1996).

The aim of the present population-based nationwide study was to determine the incidence and histological distribution of SGC, to describe the treatment modalities and patient outcome and to identify new prognostic factors for SGC. The clinical study population consisted of Finnish patients followed-up for a decade.

2. REVIEW OF THE LITERATURE

2.1. Anatomy and morphology of the salivary glands

The salivary glands in the head and neck consist of three paired, large aggregations of exocrine glandular tissue known collectively as the major salivary glands: the parotid, submandibular (submaxillary) and sublingual glands. The minor salivary glands are small, scattered nonuniformly beneath the mucosal lining of the upper aerodigestive tract. Salivary gland tissue consists of branching ducts that contain the principal secretory cells, the acinar cells. The salivary glands produce fluids that form the oral saliva, which is produced at a rate of 600-1500 ml every day (Ellis and Auclair 1996, Mayers and Ferris 2007).

The acinar cells are arranged in clusters of three to six cells (Figure 1). A tiny lumen is bordered by the acinus and the acinus itself is surrounded by a basement membrane. There are two types of acinar cells in acini, serous and mucous, whose secretions differ (Mayers and Ferris 2007). Serous cells make a thin, watery fluid that contains alpha-amylase. It begins the digestion of food during mastication in the oral cavity and digests starches. The mucous acini generate a more viscous, mucinous saliva, which contains more glycoproteins and provides a protective lubricating film on the oral mucosa (Turner and Sugiya 2002). The composition of the saliva is dependent on the relative proportion of these two cell types which varies among the salivary glands.

Contractile myoepithelial cells are located between the basement membrane and the acinar cells and they help in draining saliva through the ductal system (Barnes et al. 2005). The histologic and ultrastructural features of myoepithelial cells include both epithelium and smooth muscle, which should provide the structure with contractile properties that aid in the secretion of saliva (Hanna and Suen 1998).

The saliva is drained by a series of ducts. Intercalated duct cells are the first duct cells after the acini and are located between acinar and striated duct cells. They are very different from acinar, myoepithelial and striated duct cells as far as special or characteristic ultrastructural features are concerned. An intercalated duct drains into a striated duct, which empties into an excretory duct in the direction of the opening of the salivary gland unit (Hanna and Suen 1998, Mayers and Ferris 2007).

Basal (reserve) cells line the outer side of excretory ducts. They are relatively isomorphic and have a prominent basal cell layer and distinct basement membrane-like structures (Seifert 1991).

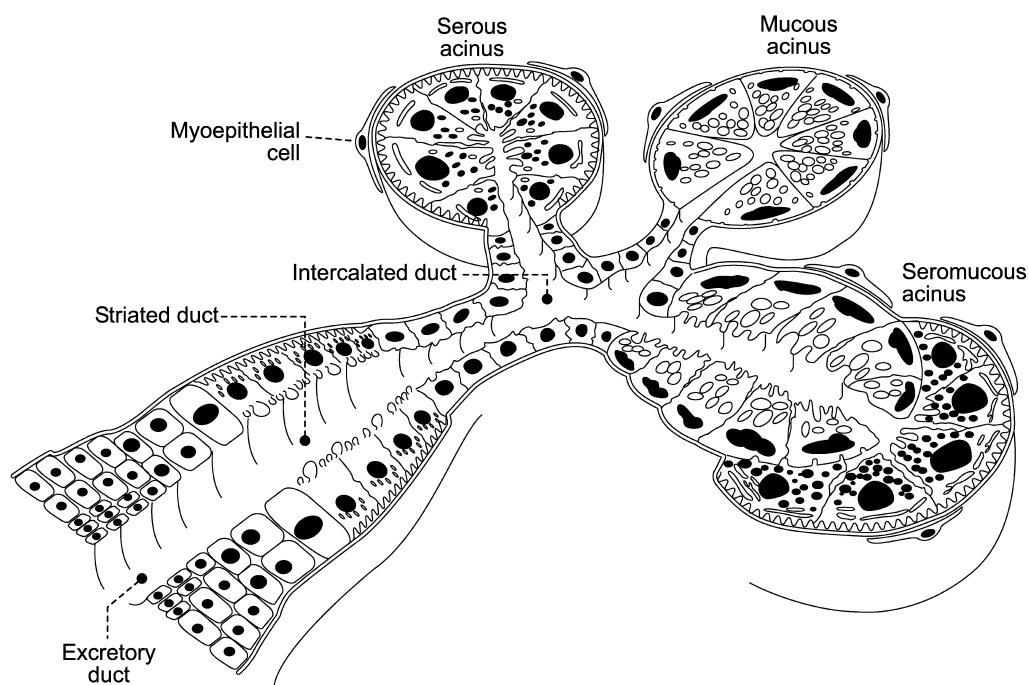


Figure 1. Structure of salivary gland acini (by Timo Kattelus, University of Turku).

The acini of the parotid gland are almost 100% serous. The close relationship between parenchymal and lymphoid tissue in the parotid gland is exceptional among salivary gland tissues and has to be considered when planning the treatment of a malignant disease of the parotid. During embryonic development the parotid is seeded with lymphoid cells that develop into several lymph nodes within and around the parenchymal tissue. The lymph nodes are often located in the superficial lobe of the gland. Lymph vessels pass to retromandibular, superficial and deep cervical lymph nodes, which has to be considered when treatment is planned (McKean et al. 1985).

The structure of the submandibular gland is largely similar to the parotid gland. Most of the acinar tissue is comprised of mucous cells and about 10% is serous. There are no lymph nodes, lymphoid nodules nor large peripheral nerves within the gland. The lymph vessels drain to prevascular and preglandular submandibular lymph nodes. The lymph vessels run straight through to subgastric lymph nodes from the dorsal side of submandibular gland (Mayers and Ferris 2007).

In the sublingual glands, both the intercalated ducts and the striated ducts are relatively short. Lymphoid tissue and large peripheral nerves are not present in the sublingual gland. The lymph vessels lead to submandibular or to deep jugular lymph nodes (Mayers and Ferris 2007).

The minor salivary glands are primarily mucus-secreting and occur mainly in the palate, nasal cavity, lips and buccal mucosa. The glands of the nasal cavity, larynx and

bronchi do not contribute to the saliva; by definition they are not salivary glands (Ellis and Auclair 1996). They are morphologically and functionally similar to the minor salivary glands of the oral cavity. There are 600 to 1000 lobules of minor salivary gland tissue dispersed within the mucosa of the oral cavity. These intraoral glands are usually clinically inconspicuous, although labial salivary lobules often can be palpated. The gland lobules are usually 1 to 5 mm in size. They are not encapsulated but are separated from one another by connective tissue beneath the mucous membrane. This feature has to be taken into account in the diagnosis of neoplasms at this site, as it may affect the management of malignant tumors (Spiro and Spiro 2001, Mayers and Ferris 2007).

2.2. Histological typing of salivary tumors

2.2.1. Classification

Salivary gland cancer (SGC) of the head and neck region is a rare cancer and it constitutes morphologically a very heterogeneous group of tumors. Classification is inconsistent and the biological behavior is highly variable ranging from low-grade to high-grade. The morphological diversity of SGC is a well-known problem and poses a challenge to the diagnosis and classification of salivary gland tumors (Eveson 1992).

Foote and Frazel published the first well-recognized histological classification of SGC in 1953 (Foote and Frazel 1953), followed by Batsakis in 1979 (Batsakis 1979). The first WHO classification was published in 1972 (Thackray and Sobin 1972) and revised in 1991 by Seifert et al. (Seifert 1991). The latest version was published in 2005 (Table 1). Currently, 24 salivary gland carcinoma entities have been described and some are also grouped by degree of malignancy. Carcinoma ex pleomorphic adenoma is classified into three types based on the degree of invasion into the capsule. The low-grade cribriform cystadenocarcinoma was originally described as a low-grade salivary duct carcinoma but has been renamed to avoid confusion with salivary duct carcinoma (Barnes et al. 2005).

The malignant salivary gland tumors of epithelial origin are showed in Table 1. The malignant tumors of nonepithelial origin are typically malignant fibrous histiocytoma, malignant schwannoma and rhabdomyosarcoma. The most common lymphoma is non-Hodgkin's lymphoma with a high degree of differentiation. Mucosa-associated lymphoid tissue (MALT) lymphomas (Roh et al. 2008) and especially marginal zone lymphomas (Anderson et al. 2009b) are associated with chronic immunosialadenitis (*i.e.*, Sjögren's syndrome). The secondary tumors of the salivary glands consist usually of metastases from primary squamous cell carcinomas or from melanomas of the skin of the head and neck region.

Table 1. WHO's histological classifications of malignant salivary gland tumors of epithelial origin, 1991 (Seifert 1991) and 2005 (Barnes et al. 2005).

Seifert 1991	Barnes 2005
Acinic cell carcinoma	Acinic cell carcinoma
Mucoepidermoid carcinoma	Mucoepidermoid carcinoma
Adenoid cystic carcinoma	Adenoid cystic carcinoma
Polymorphous low-grade adenocarcinoma (terminal duct adenocarcinoma)	Polymorphous low-grade adenocarcinoma
Epithelial-myoepithelial carcinoma	Epithelial-myoepithelial carcinoma *Clear cell carcinoma, not otherwise specified
Basal cell adenocarcinoma	Basal cell adenocarcinoma
Sebaceous carcinoma	Sebaceous carcinoma *Sebaceous lymphadenocarcinoma
Papillary cystadenocarcinoma	*Cystadenocarcinoma *Low-grade cribriform cystadenocarcinoma
Mucinous adenocarcinoma	Mucinous adenocarcinoma
Oncocytic carcinoma	Oncocytic carcinoma
Salivary duct carcinoma	Salivary duct carcinoma
Adenocarcinoma	*Adenocarcinoma, not otherwise specified
Malignant myoepithelioma (Myoepithelial carcinoma)	Myoepithelial carcinoma
Carcinoma in pleomorphic adenoma (Malignant mixed tumor)	Carcinoma ex pleomorphic adenoma *Carcinosarcoma *Metastasizing pleomorphic adenoma
Squamous cell carcinoma	Squamous cell carcinoma
Small cell carcinoma	Small cell carcinoma *Large cell carcinoma *Lymphoepithelial carcinoma
Undifferentiated carcinoma	Sialoblastoma
Other carcinoma	

* New entities in 2005.

Hematogeneous metastases are rare and they originate mainly from the lungs, kidneys or breasts (Hanna and Suen 1998). Certain benign tumors, such as the pleomorphic adenoma, basal cell adenoma, myoepithelioma and oncocytoma, may transform into the respective malignant counterparts, *i.e.*, carcinoma ex pleomorphic adenoma, basal cell adenocarcinoma, myoepithelial carcinoma and oncocytic carcinoma (Cheuk and Chan 2007).

2.2.2. Grading of salivary gland tumors

A division into low-grade and high-grade may be made for the adenoid cystic carcinoma (AdCC), the mucoepidermoid carcinoma (MEC) and the epithelial-myoepithelial carcinoma (EMC). Furthermore MEC has also an intermediate form. The previous

classifications have been criticized for not being applicable to clinical practice (Ellis and Auclair 1996). A more feasible grouping would be based on the degree of malignancy (Leivo 2006).

Some malignancies are nearly always high-grade, *i.e.*, the salivary duct carcinoma (SDC), the large cell carcinoma (LCC) and the small cell carcinoma (SmCC), whereas some are invariably low-grade, *i.e.*, the polymorphous low-grade adenocarcinoma (PLCC), the AcCC and the EMC (Mayers and Ferris 2007).

The most common high-grade carcinomas are AdCC (Seethala et al. 2007b), MEC high-grade, SDC and Ca-ex-PA. The most common low-grade (or non-high-grade) carcinomas include MEC low-grade, AcCC and PLCC (Bradley 2001).

Mendenhall et al. (2005) include, in the group of high-grade tumors, the poorly differentiated carcinoma and the anaplastic carcinoma, since they tend to appear late in life and have an aggressive biological behavior. The morphological criteria for the degree of malignancy of lymphoepithelial carcinomas have not been uniformly agreed upon. High-grade tumors are more aggressive in nature, but there are no widely accepted or validated grading systems (Barnes et al. 2005, Mendenhall et al. 2005).

The third category, presented by Leivo (2006), comprises the AdCC with a poor long-term prognosis, the myoepithelial carcinoma and the epithelial-myoepithelial carcinoma where the spectrum of behavior is not well known yet (Leivo 2006).

2.3. Immunohistochemistry

Immunocytochemical techniques offer additional information for morphological classification, cellular differentiation and cell proliferation of salivary gland tumors, some of which have prognostic significance. This information is useful for the selection of the treatment of certain tumors. Immunohistochemistry determines the expression of antibodies to cellular proteins in tissue specimens. The site of this expression is associated with cell structures or cell functions.

Immunohistochemistry was first developed for frozen tissue specimens and later for formalin-fixed and paraffin-embedded specimens. Fixation and preparation of tissue specimens are the essential parts of the immunohistochemical technique; they influence the results and may explain various features reported by different investigators. Some antigens are preserved better in one fixative than in another. However, formalin fixation is the standard for most tissues in most laboratories.

For analysis of cell differentiation, a variety of new immunohistochemical markers for different cell types have been introduced during the recent years. An appropriate combination of them aids diagnostics and differential diagnostics of diverse histological entities. There are three main types of epithelial cells of the salivary glands which can

be distinguished by tumor markers: acinic cells, ductal cells and myoepithelial cells (de Araujo et al. 2000).

Prudence has to be considered when connecting an immunohistochemical marker with a histological type, since there is seldom a marker that is specific for only one type of tumor. Some illustrative examples of this circumstance when the markers in most cases turn positive are the androgen receptor which is expressed in salivary duct carcinoma (Laurie and Licitra 2006, Williams et al. 2007) and the transmembrane tyrosine kinase receptor C-KIT (CD117) in adenoid cystic carcinoma (Ettl et al. 2008). Functional markers have been associated with survival, *e.g.*, expression of Ki-67 is a well-known prognostic marker in salivary gland cancer (Ben-Izhak et al. 2008).

2.3.1. Ki-67

The proliferative activity of a tumor is defined as the growth fraction. This can be determined by counting the number of cells in the nonresting stage of the cell cycle relative to the total number of cells. The *MKI67* gene, which is located in chromosome 10, encodes the Ki-67 protein. The Ki-67 antigen is a nonhistone protein expressed in cycling cells in G1 phase, S phase, G2 phase and during mitosis, but not in G0 phase of resting cells (Gerdes et al. 1983, Pich et al. 2004).

Originally, the Ki-67 antigen could be detected only in frozen sections since the reaction was degraded by formalin fixation. The method has been improved and currently the monoclonal antibody MIB-1 is used; it detects the recombinant fragments of the Ki-67 antigen and works in formalin fixed, paraffin-embedded tissues. The antibody reacts selectively with the nuclei of proliferating cells (Skalova et al. 1994a).

Ki-67 immunoreactivity is a prognostic factor in numerous human cancers (Tubiana and Courdi 1989), including head and neck cancer (Pich et al. 2004) and some salivary gland carcinomas (Dodd and Slevin 2006): MEC (Skalova et al. 1994a, Kiyoshima et al. 2001), AcCC (Skalova et al. 1994b and Hellquist et al. 1997), AdCC (Nordgård et al. 1997) and Ca-ex-Pa (Xin and Paulino 2002), but its value as a prognostic factor in this histologically unhomogenous group of carcinomas has not been fully elucidated.

Counting of mitotic figures is the traditional way to assess tumor proliferation. With light microscope, proliferation can be recognized without special devices (van Diest et al. 1998). In an approach introduced by Haapasalo et al. (1989), the number of mitotic figures is corrected for the area percentage of tumor tissue, which yields the mitoses per volume (MV) index. An application of this method can be used in counting of Ki-67 positive cells.

2.3.2. p53

The occurrence and location of mutated forms of antigens to the tumor suppressor gene p53 associated protein have been extensively studied to understand tumor biology.

The gene is TP53 located on the short arm of chromosome 17. It encodes the tumor suppressor protein p53, the normal function of which is to modulate the transcription of genes that manage the major defences against tumor growth. This modulation involves cell cycle arrest, apoptosis, maintenance of genetic integrity, inhibition of angiogenesis and cellular senescence. Failure of p53 to function properly results in uncontrolled net growth, a feature of cancer cells (Vogelstein et al. 2000, Vogelstein and Kinzler 2004).

The p53 protein does not function correctly in most human cancers (Hollstein et al. 1994, Selivanova and Wiman 2007). The most common mechanism of p53 pathway disruption is through a point mutation. p53 may also be inactivated indirectly through an infection with DNA tumor viruses: the products of these viruses, *e.g.*, the E6 protein of the human papilloma virus, bind to p53 and inactivate it. p53 is also inactivated as a result of alterations in genes whose products interact with p53 or transmit information to or from p53, *e.g.*, the MDM2 gene. Under normal conditions, MDM2 is the most important negative regulator of p53 (Vogelstein et al. 2000, Vogelstein and Kinzler 2004, Whibley et al. 2009). The mutant p53 gene encodes proteins with a prolonged half-life; as a consequence, these mutations frequently lead to a relative overexpression of the p53 protein (Pfeifer and Besaratinia 2009).

The complex cellular feedback system, or the p53 network, is normally inactive and becomes activated only when cells are stressed or damaged (Chari et al. 2009). Abnormal cells cause a threat to the organism, as they may contain mutations or exhibit abnormal cell cycle control and in this manner present a greater risk of becoming cancerous (Vogelstein et al. 2000).

Mutations of p53 are often associated with a poor prognosis. p53 has been studied also in SGC but the association between the activity of p53 and patient survival has not been clear (Soini et al. 1992, Gallo et al. 1995, Kärjä et al. 1997, Zhu et al. 1997, Nagao et al. 2002, Doi et al. 1999, Kiyoshima et al. 2001, Lim et al. 2003, Alves et al. 2004).

2.3.3. MMP

Matrix metalloproteinases (MMPs) are zinc-dependent neutral endopeptidases that play a key role in the physiologic degradation of the extracellular matrix (ECM) in situations like angiogenesis, tissue repair and tissue morphogenesis. The MMPs have been associated with tumor growth and oncogenesis in different organs. High levels of MMPs are generally associated with the invasive properties of cancer and their impact on patient prognosis has been studied widely in different cancer types (Ala-aho and Kähäri 2005, Vihinen et al. 2005). They break down all components of the ECM, including collagens, elastin, proteoglycans, laminin and fibronectin (De et al. 2005). They also cleave several non-matrix proteins, including growth factors, cytokines, chemokines and their receptors, in this manner regulating cell growth and inflammation (Nagase and Woessner 1999). The gene family can be divided into the following subgroups by

substrate specificity and function: collagenases, matrilysins, gelatinases, stromelysin, membrane-type MMPs and other MMPs (Visse and Nagase 2003).

Collagenase-1 (MMP-1) and collagenase-3 (MMP-13) are two of the principal proteinases capable of cleaving native fibrillar collagens, which are the most abundant structural components of human connective tissue. The ability to degrade connective tissue is apparently crucial for the invasion and metastasis of neoplasia (Ala-aho and Kähäri 2005, Vihinen et al. 2005). Strong expression of MMP-1 has been associated with a poor prognosis of patients with colorectal, esophageal, gastric and oral carcinomas and with metastatic melanoma and pancreatic adenocarcinoma (Ala-aho and Kähäri 2005, Vihinen et al. 2005, Nikkola et al. 2002, Nikkola et al. 2005, Murray et al. 1996, Murray et al. 1998a, Murray et al. 1998b, Ito et al. 1999). MMP-13 is expressed by invading tumor cells in squamous cell carcinoma (SCC) of the head and neck as in vulvar SCC cells (Johansson et al. 1997, Johansson et al. 1999). Similarly, a strong expression of MMP-13 and a poor patient prognosis have been linked together in colorectal, esophageal and gastric carcinomas (Ala-aho and Kähäri 2005, Vihinen et al. 2005) and HNSCC (Ala-aho and Kähäri 2005, Vihinen et al. 2005, Luukkaa et al. 2006).

Matrilysin-1 (MMP-7) is mainly expressed in the epithelial cells of various glandular structures of the endometrium, small intestine, breast, parotis, pancreas, liver, prostate, dermis and bronchus, and in epithelial tumors of the gastrointestinal tract, prostate and breast (Saarialho-Kere et al. 1995, Vihinen et al. 2005). MMP-7 contributes to the intestinal mucosal defense by activating antibacterial peptides, defensins (Wilson et al. 1999). Besides ECM components, MMP-7 processes cell surface molecules, *e.g.*, pro- α -defensin, Fas-ligand, pro-tumor necrosis factor (TNF)- α , and E-cadherin (Visse and Nagase 2003). Strong expression of MMP-7 has been associated with poor patient prognosis in pancreatic adenocarcinoma (Yamamoto et al. 2001) and esophageal squamous cell carcinoma (Yamashita et al. 2000). Correspondingly, a stronger expression has also been linked with hepatocellular cancer recurrence (Yamamoto et al. 1999) and lymph node metastasis of gastric carcinoma (Yamashita et al. 1998).

Gelatinase B (MMP-9) degrades basement membrane collagens which appears to be crucial for metastatic tumor cell penetration and invasion (Nagase and Woessner 1999). Gelatinases have also been linked to tumor angiogenesis, especially gelatinase B. Furthermore, strong expression of MMP-9 carries significant negative prognostic value to patients with head and neck squamous cell carcinomas, breast, colorectal, gastric, hepatocellular, ovarian, pancreatic, prostate, renal and non-small cell lung cancers, and metastatic melanoma (Ala-aho and Kähäri 2005, Vihinen et al. 2005, Nikkola et al. 2005, Riedel et al. 2000).

MMP expression in salivary gland cancer has been studied to some extent (Azuma et al. 1993, Soini and Autio-Harmainen 1993, Freitas et al. 2004, Kayano et al. 2004, Nagel et al. 2004, Westernhoff et al. 2005), but the role of MMP expression as a prognostic indicator in this histologically heterogenous group of carcinomas has not been extensively

or systematically examined. de Vicente et al. (2008) reported that the invasiveness and prognosis of high-grade salivary gland cancers may depend on their MMP-9 expression profile.

2.4. Angiogenesis

Angiogenesis refers to the development of new blood vessels from preexisting vasculature. This process is essential for normal and neoplastic tissue growth (Folkman 1990). Angiogenesis is a precisely controlled series of physiological cellular events, including vascular initiation, formation, maturation, remodeling and regression, which are controlled and modulated for certain tissue. Pathological angiogenesis, on the other hand, is inadequately controlled. The initiation and formation stages do occur, but the vessels rarely mature, remodel or regress in disease (Staton et al. 2009). Every multiplication of a tumor cell population is preceded by an increased number of new capillaries (Folkman 2006a). Without appropriate neovascularization, neoplasms exceeding a certain size become necrotic (Folkman 1990). Novel targeted therapies utilizing antiangiogenic agents aim at reducing the blood flow to the cancer cells; antiangiogenic treatment can slow down tumor growth and reduces formation of metastasis (De Paepe 2009, Zahorowska et al. 2009).

Increased angiogenesis is characteristic of more than 50 diseases, *e.g.*, cancer, coronary heart disease, rheumatoid arthritis, psoriasis and diabetes mellitus (Folkman 2006a). Angiogenesis is crucial for the growth, invasion and metastasis of solid neoplasms. Angiogenesis in epithelial cancers of different organs has been found to associate with prognosis in more than 100 studies (Makrilia et al. 2009) and angiogenesis may have prognostic value for patients with head and neck cancer (Ascani et al. 2005, Erovic et al. 2005). Furthermore, in the adenoid cystic carcinoma the overexpression of vascular endothelial growth factor (VEGF) is related to increased angiogenesis (Zhang et al. 2005), but not with certainty in head and neck squamous cell carcinoma (Tae et al. 2000).

The assessment of angiogenesis is influenced by the pattern of vessel formation. The formation of new blood vessels, angiogenesis, by proliferation of new capillaries from already existing vessels differs from vasculogenesis, which is the formation of blood vessels *de novo* from angioblasts during embryogenesis (Carmeliet and Jain 2000). Microvessel density may be considered as an indirect indicator of neo-angiogenesis and thus to causally precede or accompany malignancy (Folkman 1990). Vessels in tumor samples can be identified with different endothelial cell-specific antibodies, some of which recognize antigens related to factor VIII, CD31, or CD34 (Tanigawa et al. 1996, Meert et al. 2002) and they may be further analyzed by computer-assisted quantitative image analysis (CAQIA) (Laitakari et al. 2004). In invasive breast cancer, CD34 yields higher microvessel values than CD31 or factor VIII (Martin et al. 1997) and is more sensitive and specific than factor VIII for staining endothelial cells induced

by tumor neovascularization in gastric carcinoma (Tanigawa et al. 1996). In head and neck carcinoma the role of increased angiogenesis has been disputed in studies utilizing immunohistochemical analysis with the anti-CD34 monoclonal antibody (Erovcic et al. 2005, Ascani et al. 2005), while in laryngeal cancer the same method has shown increased angiogenesis (Laitakari et al. 2003 and 2004).

The number of vessels in tumor samples can be identified with different techniques: vessel counting in areas with high numbers (Weidner 1995), point counting (Pazouki et al. 1997) or vessels visualized by the endothelial cell-specific antibody plus CAQIA (Laitakari et al. 2004). CAQIA is a feasible application for combining morphological and functional features of a tissue analysis (Laitakari et al. 2004) and it yields information also on vessel size, vessel shape and vessel staining intensity. Comparisons of results within the same study are more reliable when done with computer-assisted morphometry than visually or by histoscore determinations (Barth et al. 1996). In addition to improved reproducibility and objectivity CAQIA allows the study of large numbers of samples.

2.5. HER-2

The c-ErbB-2 gene, also known as HER2/neu, codes for the ErbB2/human epidermal growth factor receptor-2 (HER-2). It is a member of the ErbB protein group family that is also known as the epidermal growth factor receptor, EGF receptor (EGFR) family.

HER-2 is overexpressed on the surface of ductal carcinoma cells of the breast in 20% to 30% and in 15–20% of all breast cancers and is associated with a poor patient prognosis (Nabili et al. 2007, Ross et al. 2009).

New, specific treatment modalities have been developed and the results have been promising, indeed. Trastuzumab (Herceptin[®], Genentech Inc., San Francisco, CA, USA) is widely used clinically. It is a monoclonal antibody which binds with high affinity to the extracellular domain of HER-2. Trastuzumab inhibits the proliferation of tumor cells which overexpress HER-2 (Daniele and Sapino 2009). The drug increases the disease-free interval and prolongs overall survival for patients with ductal carcinoma of the breast. Other novel HER2-targeting agents include pertuzumab (Omnitarg[®], Genentech Inc., San Francisco, CA, USA), a monoclonal antibody which inhibits the dimerization of HER-2 with other HER receptors. Lapatinib (Tykerb/Tyverb[®], GlaxoSmithKline, Research Triangle Park, NC, USA) is a dual small molecule tyrosine kinase inhibitor targeting HER-1 (EGFR) and HER-2 (Ross et al. 2009).

In SGC, HER-2 is often expressed in SDC (Williams et al. 2007). HER-2 positivity has also been connected to other histological types (Laurie and Licitra 2006). An association between HER-2 expression and the prognosis of patients with epithelial-myoepithelial carcinoma (Cho et al. 1995) and with MEC has been reported (Press et al. 1994).

2.6. Epidemiology

Salivary gland carcinomas are uncommon neoplasms. They account for only 3–6% of all head and neck malignancies (Spiro et al. 1989, Kane et al. 1991, Shah and Patel 2003, Finnish Cancer Registry 2009). The percentage of malignant tumors out of all salivary gland neoplasms varies between 21.0% and 36.8%. The proportion of malignant parotid tumors is 17–34%, of submandibular gland tumors 30–55 % and of minor salivary neoplasms 40–85%. Tumors of the sublingual glands are often united with minor salivary glands. The proportion of malignant neoplasms of all sublingual tumors is 80–90% (Eveson and Cawson 1985, Seifert et al. 1986, Spiro 1986, Ellis et al. 1991, Barnes et al. 2005).

The incidence of malignant salivary gland neoplasms varies significantly in different parts of the world, from 0.4 to 2.6 new cases/100 000 person-years (Koivunen et al. 2002, Östman et al. 1997, Pinkston and Cole 1999, Sun et al. 1999). The geographic variation may partly be due to different methods of registration and epidemiological features. The Surveillance, Epidemiology, and End Results (SEER) and American Cancer Society registries record salivary gland cancers in the anatomical site of origin, *e.g.*, oral cavity and pharynx, rather than within a single, organ-specific salivary gland entity (Witt 2005). The latest incidence figure from the USA is 1.2/100 000 (2000-2004) (SEER 2010). Very high incidence rates have been reported in Greenland (Albeck et al. 1992) and among the Canadian Arctic inuits, 13.5/100 000 person-years; lymphoepithelial carcinomas formed no less than 25% of malignancies. Since 1980s the relative frequency has declined (Schaefer et al. 1975, Hildes and Schaefer 1984, Ellis and Auclair 1996). The high risk of SGC persists after migration to a low incidence area, which indicates that genetic or environmental factors gained early in life are etiologically important (Boysen et al. 2008).

The incidence of SGC in Finland is low and SGC account for approximately 0.3 % of all cancers. SGC is among the six most common malignant neoplasms of the head and neck in Finland (Finnish Cancer Registry 2009). The age-adjusted incidence rates during the years 1960-2005 ranged from 0.5 to 0.8 per 100 000 person-years for males and from 0.5 to 0.7 for females. The latest statistics from 2010 notes the figures 0.8 for males and 0.6 for females. This equals an absolute number of 63 new cases in 2008 (Finnish Cancer Registry 2010).

2.7. Etiology

Ionizing radiation and certain occupational exposures are associated with an increased incidence of salivary gland cancer. Serum or tissue markers associated with a risk for neoplastic transformation have not been reported.

2.7.1. Radiation

The role of the etiological factors for the development of salivary gland cancer is not well understood, but exposure to ionizing radiation from various sources is clearly

associated with these tumors (Takeichi et al. 1983, Little 2001). Cellular, mobile and cordless phones have been under a strong focus regarding a possible association with salivary gland tumors. Most studies have not found any association (Johansen et al. 2001, Auvinen et al. 2002, Elwood 2003, Hardell et al. 2004, Kundi et al. 2004). In one study, an increased risk ratio was reported for ipsilateral regular use of phones at 5 and 10 years (OR 1.5), although the 10-year risk was based on small number of patients (Sadetzki et al. 2008a).

2.7.2. Occupation, lifestyle and nutrition

An increased risk of salivary gland cancer has been associated with certain occupations, *e.g.*, some types of woodworking, plumbing, asbestos mining, rubber manufacturing, among hairdressers and in personnel employed in beauty shops, but the clinical significance has by no means been established (Swanson and Belle 1982, Horn-Ross et al. 1997a). Swanson and Burns (1997) reported that a very high level of exposure to cigarette smoking, ≥ 80 pack-years, is associated with salivary gland cancer. Tobacco and alcohol together are reportedly a risk factor for SGC among males but, curiously enough, not among females (Horn-Ross et al. 1997b).

2.7.3. Cutaneous tumors

The risk ratio for major salivary gland cancer among patients with skin cancer is 4.3 and among males with skin cancer no less than 13.7 (Spitz and Batsakis 1984, Spitz et al. 1985). The biological relationship between cutaneous neoplasms and neoplasms of the major salivary glands may well be related to their common embryologic derivation, histogenesis, and histological composition (Batsakis and Brannon 1981, Spitz and Batsakis 1984). The epidemiologic similarities between salivary gland cancer and nonmelanoma skin cancer could also be related to exposure to solar ultraviolet radiation (Spitz et al. 1990, Nagler and Laufer 1997). The possible mechanisms include gene alterations, such as increased p53 mutations, or altered host responses allowing progression of preneoplastic or neoplastic alterations or decreased immunological defence mechanisms (Spiro and Spiro 2001, Barnes et al. 2005).

2.7.4. Hormones

Endogenous hormone receptors, *e.g.*, of estrogen, progesterone and androgen, have been identified in normal and neoplastic salivary glands, but no clear etiologic nor prognostic pattern has emerged (Barnes et al. 1994a, Jeannon et al. 1999, Dori et al. 2000, Nasser et al. 2003).

2.7.5. Viruses

Some viruses have been connected in the pathogenesis of salivary gland cancer. There is a clear link between Epstein-Barr virus (EBV) infection and lymphoepithelioma-like carcinomas; the association is approximately 100% in Eskimo and Asian populations

(Iezzoni et al. 1995). EBV has also been detected in undifferentiated salivary gland carcinoma (Hamilton-Dutoit et al. 1991, Gallo et al. 1994). This implies that a specific EBV strain may have special oncogenic potential in Greenland and Asia than in other parts of the world (Tsai et al. 1996). In contrast, Atula et al. (1998) did not identify EBV, human herpesvirus 8, human papilloma virus nor cytomegalovirus in salivary gland cancer.

2.8. Histology and clinical features

2.8.1. Mucoepidermoid carcinoma (MEC)

According to many reports, MEC is the most common carcinoma of the salivary glands and accounts for 30% of all salivary gland malignancies (Myers and Ferris 2007). Usually it occurs in the parotid gland. There is a 3:2 female predilection (Barnes et al. 2005). The tumor mass is usually slowly enlarging, often over several years and is painless. In intraoral sites superficial neoplasms may exhibit a blue-red color and mimic a mucocele or vascular lesion (Shah and Patel 2003, Bhattacharyya and Fried 2005, Myers and Ferris 2007). According to the multicellular theory, mucoepidermoid tumors develop in the excretory duct cells while the reserve cell theory proposes that the origin is in the reserve cell of the excretory duct (Hanna and Suen 1998). Prognosis is influenced by the degree of malignancy, tumor grade (Mendenhall et al. 2005) and stage, and the gender and age of the patient (Pires et al. 2004).

A histological grading of mucoepidermoid carcinomas has been suggested (Spiro et al. 1978, Evans 1984, Nascimento et al. 1986, Batsakis 1994, Brandwein et al. 2001). Another system created by Auclair, Goode and Ellis (Auclair et al. 1992, Ellis and Auclair 1996) has been repeatedly used (Goode et al. 1998, Guzzo et al. 2002, Aro et al. 2008).

The histological grading of mucoepidermoid carcinoma has been related to prognosis. The 5-year survival rate of patients with low and intermediate/high-grade cancer is about 90% and 50% respectively, and the 10-year rates 90–97% and 23–42%, respectively (Spiro et al. 1978, Evans 1984, Guzzo et al. 2002).

2.8.2. Adenoid cystic carcinoma (AdCC)

The adenoid cystic carcinoma is the second most common salivary gland cancer and accounts for 20% of all salivary gland malignancies (Myers and Ferris 2007). About 75% arise in the minor and 25% in major salivary glands (Spiro et al. 1974, Shah and Patel 2003). The clinical behavior of AdCC is characterized by a high rate of local recurrence and distant metastases (Myers and Ferris 2007). According to the reserve cell theory, AdCC originates from the reserve cell of the intercalated duct (Dardick and Burford-Mason 1993).

The histological growth patterns are glandular (cribriform), tubular and solid. In AdCC, grading as a prognostic factor has been problematic (Spiro and Huvos 1992), although most of the literature supports the pattern-based histologic grade as a prognostic indicator, where the presence and quantity of the solid form of AdCC is important (Ellis and

Auclair 1996, Kokemueller et al. 2004a, da Cruz Perez et al. 2006). If the tumor consists of more than 30% of solid component, the tumor is more aggressive than if the fraction of solid component is less (Szanto et al. 1984, da Cruz Perez et al. 2006). Perineural or perivascular invasion without a stromal reaction is very characteristic of AdCC (Spiro et al. 1974, Seifert 1991, Barnes et al. 2005).

The tumor grows slowly, and clinical attention is characteristically paid to the pain that originates from perineural tumor invasion. Facial nerve paresis occurs sometimes (Spiro et al. 1974, Barnes et al. 2005). The occurrence of distant metastasis ranges from 20 to 60%; the lung, bone, brain and liver are the most common sites of distant metastasis (Myers and Ferris 2007).

The 5-year survival of patients with distant metastases is 20 % (Bradley 2004). The local recurrence rate ranges from 16% to 85% in several series. Chen et al. (2008) studied 46 patients with AdCC of whom 12 (26%) had a late recurrence after a 5-year follow-up. The 5-, 10- and 15-year survival rates are disappointing, about 75%, 40% and 8–25% respectively (Spiro et al. 1974, Szanto et al. 1984, Hamper et al. 1990, Kokemueller et al. 2004a).

2.8.3. Acinic cell carcinoma (AcCC)

Acinic cell carcinoma is an uncommon low-grade salivary gland malignancy representing 2–4% of all salivary gland neoplasms. Most of these tumors, 80% to 90%, occur in the parotid gland. Women are affected more often than men at a ratio of 2:1 (Myers and Ferris 2007). According to the multicellular theory, acinous cell tumors originate from acinar cells. Most researchers maintain, however, in line with the reserve cell theory, that acinous tumors originate from the reserve cell of the intercalated duct (Dardick and Burford-Mason 1993).

Acinic cell carcinoma is typically a slowly growing, solitary, unfixed mass in the parotid region. One fifth of the patients experience pain and 3% to 10 % of the patients develop facial nerve paresis (Barnes et al. 2005). The duration of symptoms is usually 2 years but sometimes the history may extend over several decades (Ellis and Corio 1983, Lewis et al. 1991).

The histologic patterns do not correlate well with the biological behavior or prognosis; the TNM stage is a better predictor of outcome. AcCC behaves more aggressively in the major salivary glands than in minor glands. The disease is most aggressive when it occurs in the submandibular gland (Hoffman et al. 1999). The recurrence rate is about 35% (Barnes et al. 2005). A well known problem is that of late recurrences, and follow-up of at least 10–20 years is recommended (Eneroth et al. 1966, Wahlberg et al. 2002). Distant metastases develop in 16% of the patients (Lewis et al. 1991).

The 5-year overall survival rate of patients with acinic cell carcinoma is about 80% and the 10-year rate 50-85% (Spitz and Batsakis, Spiro et al. 1989). In a study of Hoffman

et al. (1999), the 5-year disease specific survival was 91% and overall survival 83%. Wahlberg et al. (2002) found that the relative 10- and 20-year survivals were 88% and 83%, respectively.

2.8.4. Salivary duct carcinoma (SDC)

Salivary duct carcinoma is a highly malignant epithelial tumor. Histologically, rather large cell aggregates are formed which resemble distended salivary ducts. SDC resembles high-grade ductal carcinoma of the breast, and 60% of the patients have perineural spread (Seifert 1991, Barnes et al. 2005). The histogenesis is derived from the excretory duct of reserve cells (Dardick and Burford-Mason 1993). SDC constitutes about 9% of all salivary gland malignancies and occurs most often (78%) in the parotid gland (Myers and Ferris 2007). The male-female ratio is 4:1 (Barnes et al. 1994b, Barnes et al. 2005).

Patients usually present with a rapidly growing tumor that may fluctuate in size. Some patients have long clinical histories. Pain and facial nerve paresis may be present. At the time of diagnosis, about two-thirds of the patients present with T3 or T4 tumors and the disease has spread to the lymph nodes in 56% of patients (Barnes et al. 1994b, Jaehne et al. 2005). Along with the aggressive clinical course, early distant metastasis is typical (Barnes et al. 2005).

In a review of 104 patients by Barnes et al. (1994b) and 50 by Jaehne et al. (2005), local recurrence occurs among 33% to 48% of the patients and about 48% develop distant metastasis into the lungs, skeleton, liver and brain (Barnes et al. 1994b, Jaehne et al. 2005). At least 65% of patients die of their disease, usually within 4 years (Barnes et al. 1994b).

2.8.5. Epithelial-myoepithelial carcinoma (EMC)

Epithelial-myoepithelial carcinoma of the salivary gland is a rare, low-grade malignant neoplasm (Donath et al. 1972). The EMCs comprise approximately 1% of all salivary gland tumors (Myers and Ferris 2007).

Usually (60-75% of the instances) EMC is located in the parotid gland. There is a female preponderance at a ratio of 2:1 to males (Ellis and Auclair 1996, Barnes et al. 2005). These tumors exhibit a wide range of biological behavior, since high-grade or dedifferentiated EMCs (DEMC) have been described, although rarely (Seethala et al. 2007a). EMC is frequently associated with other salivary gland carcinomas to form what is called hybrid tumours, and may also share histological features characteristic of other salivary gland tumors, *e.g.*, adenoid cystic carcinoma (Chetty et al. 2000, Nagao et al. 2002).

Other subtypes have also been described, *e.g.*, oncocytic EMC (OEMC), EMC rising in a pleomorphic adenoma (Harada 2000, Alos et al. 1999, Lewis et al. 2001, Savera and Zarbo 2004), double clear EMC and EMC with myoepithelial anaplasia (Seethala et al. 2007a). Palisading and degenerative Verocay-like changes have also been described (Seethala et al. 2007a).

Patients usually present with a painless, slow-growing mass in the parotid gland, but also rapid growth and facial nerve palsy with pain may occur, if the tumor is of high grade. Approximately 40% of patients experience a local recurrence and 14% regional lymph node metastasis; metastasis to the lung, liver and kidney occur also (Barnes et al. 2005, Myers and Ferris 2007). The 5-year overall survival rate is 80% and the 10-year rate 72% (Fonseca and Soares 1993).

Table 2. Histological distribution of salivary gland cancer.

Study, Origin	N	Site	MEC %	AdCC %	ADC %	AcCC %	Most frequent histological types
Östman et al. 1997, Sweden	2557	All	19	20	13	12	AdCC and MEC, all diagnoses from cytology
Wahlberg et al. 2002, Sweden	2465	Major	21	21	17	15	AdCC and MEC
Spiro 1985, USA, New York	1278	All	34	22	18	7	MEC is the most frequent, 62% of those in parotis
Bhattacharyya and Fried 2005, Boston, USA	903	Parotid	41	20	21	11	MEC
Terhaard et al. 2004, Utrecht, Netherlands	565	All	16	26	23	12	AdCC and ADC, WHO 1972 classification
Eneroth 1976, Sweden	528	Parotid gland, submandibular gland and minor salivary glands of palate	24	23	11	12	AdCC and MEC
Fitzpatrick & Theriault 1986, Toronto, Canada	403	All	21	22	17	6	AdCC and MEC
Therkildsen et al. 1998, Denmark	251	All	16	35	12	6	AdCC
Renehan et al. 1996, Houston, USA	244	Minor salivary glands	16	31	13	9	AdCC
Vander Poorten et al. 1999, 2000 and 2000 in book	32	Submandibular	0	50	9	13	AdCC
2002, Netherlands, Results computed from	55	Minor	16	40	16	5	AdCC
Vander Poorten et al. 1999, 2000 and 2000	135	Parotid	10	13	33	20	ADC
	222	All sites	10	25	26	15	AdCC and ADC
Satko et al. 2000, Slovakia	197	All	27	33	18	20	AdCC and MEC
Eveson and Cawson 1985, Britain	155	Minor salivary glands	19	28	26	4	AdCC and ADC
Kokemueller et al. 2004b, Hannover, Germany	155	All	27	51	8	1	AdCC
Lima et al. 2005, Rio de Janeiro, Brazil	150	Parotid	32	12	14	14	MEC
Beckhardt et al. 1995, Houston, USA	116	Minor salivary glands of the palate	16	29	19	1	AdCC
Hocwald et al. 2001, Detroit, USA	78	Major salivary glands	36	20	14	9	MEC
Jansisyanont et al. 2002, Baltimore, USA	61	Minor	54	11	5	5	MEC
Koivunen et al. 2002 Oulu, Northern Finland	40	All	30	28	8	20	MEC

AdCC: adenoid cystic carcinoma, MEC: mucoepidermoid carcinoma, ADC: adenocarcinoma, AcCC: acinic cell carcinoma

2.9. Worldwide histological distribution of salivary gland cancer

The reported histological distribution of SGC varies by study. This may be explained by inter-cohort variations and registration bias. Also, the histological criteria have changed several times. Nevertheless, there are remarkable interregional differences (Table 2). MEC is generally regarded as the most common histological type worldwide (Spiro 1985) and it is remarkable that in some studies AdCC is as common as MEC (Wahlberg et al. 2002, Östman et al. 1997).

2.10. Diagnostic aspects

A salivary gland neoplasm must be considered in any patient who presents with a painless, slowly growing swelling at the site of the parotid, submandibular or sublingual glands. Especially at the early stage, malignant and benign tumors are clinically indistinguishable. The overall detection rate for a malignant parotid gland tumor based on clinical findings is approximately 30% (Wong 2001). The mass may have been present for years in a case of a benign tumor or a low-grade salivary gland cancer. It is remarkable that adenoid cystic carcinoma usually present as a slow-growing mass. Inflammatory disease may be associated with pain and intermittent swelling. Obstruction in the parotid gland is much less frequent than in the submandibular gland; thus, a painful mass in the parotid gland may be a sign of malignant disease (Spiro and Spiro 2001). Although both benign and malignant tumors may present with pain, this is not common (Witt 2004). Finding indicating malignancy are rapid enlargement, palpable cervical lymph nodes, paresis of all or part of the facial nerve, presence of associated lymphadenopathy and fixation of the tumor to the overlying skin or deep structures (Spiro and Spiro 2001, Wong 2001, Shah and Patel 2003). Salivary gland cancer often spreads by infiltrating into the surrounding structures as nerves, bone, muscles and skin. Parotid gland cancer may infiltrate the facial nerve and invade perineurally to the base of the skull. Regional lymph node involvement is an important route for metastasis in most histological types of tumors.

2.10.1. Parotid glands

The average age of the patients with parotid gland cancer is 55 to 60 years and the tumors are distributed evenly between the genders. Before appearance, the duration of symptoms varies typically from 4 to 8 months (Eisele and Kleinberg 2004). Most parotid cancers arise in the superficial lobe (approximately 90% of patients). Initially the tumor presents as a rubbery nodular mass (Shah and Patel 2003). According to Witt (2004), 10-15% of the patients present with pain, and facial nerve paresis occurs in 10-20% of the patients, a sign associated with a poor prognosis. The deep portions of the tumors expand as pharyngeal swellings in the site of the palatine tonsil and appear clinically as a peritonsillar mass in a 5% of the patients (Graney et al. 1998, Kane et al. 1991). Sensory loss and trismus may result from tumor involvement in the infratemporal fossa. In contrast to squamous cell carcinomas, regional lymph node involvement is generally infrequent (Shah and Patel 2003). Cervical lymph node metastases occur in 10-20% and

periparotid lymph node metastases in 6% of patients (Eisele and Kleinberg 2004, Witt 2004). Pleomorphic adenoma may undergo malignant transformation (carcinoma ex pleomorphic adenoma), especially among patients with a long-standing mass (Batsakis 1979, Lewis et al. 2001).

2.10.2. Submandibular glands

The average age of the patients at presentation is 55–60 years. There is a slight male-to-female preponderance of 1.2:1. Malignant tumors in the submandibular glands constitute usually asymptomatic masses; pain is present in 6-7% of the patients. The duration of symptoms is generally approximately 6 months (Bissett and Fitzpatrick 1988). Submandibular tumors with a history exceeding 5 years are usually low-grade MEC or Ca-ex-PA (Eisele and Kleinberg 2004). Weakness or numbness of the tongue suggests involvement of the hypoglossal or lingual nerve (Witt 2004). Pathologic lymph nodes may be present, in addition to the primary tumor, in 14-16% of the patients (Bissett and Fitzpatrick 1988).

2.10.3. Minor salivary glands

Generally, a malignant tumor of the minor salivary glands presents as a painless submucosal mass in the oral cavity and, sometimes, also at other sites of the head and neck region, e.g., the nasal cavity (Spiro 1986, Spiro et al. 1991, Waldron et al. 1988). They are seldom ulcerated, but when the overlying mucosa has been traumatized, the clinical situation mimics squamous cell carcinoma (Batsakis 1979). The duration of symptoms is usually about 6 months, but 30% of patients have had them longer than 1 year. The symptoms depend on the site of the tumor (Batsakis 1979, Eisele and Kleinberg 2004).

2.10.4. Sublingual glands

Sublingual gland cancer presents as a palpable submucosal mass of the anterior floor of the mouth; about 15% of the patients have local pain (Eisele and Kleinberg 2004).

2.10.5. Clinical examination

Regarding salivary gland malignancies, the problem is that often malignancy can not be assessed clinically. Locoregional staging of malignant tumors is based on clinical examination in combination with imaging. For lesions in the superficial parotid and the submandibular gland, sonography and fine needle aspiration cytology (FNAC) are a suitable combination for initial assessment (Lee et al. 2008). Contrast enhanced computer tomography (CT) or/and magnetic resonance imaging (MRI) of the primary site and the neck and carried out in selected cases (Okahara et al. 2003). The goal of imaging studies is to define precisely the location and extent of the tumor and to identify whether the lesion is intraglandular or has extraglandular extension (Shah and Patel 2003). Computed tomography of the chest should be performed to exclude the possibility of distant metastases, if needed (Bradley 2001). The risk of distant metastases is associated with

tumor grade and is most common in patients with adenoid cystic carcinoma, high-grade mucoepidermoid carcinoma, salivary duct carcinoma and tumors of the submandibular gland, posterior part of the tongue and the pharynx (Bradley 2001).

Often the cervical lymph nodes are further studied with sonography, sometimes complemented with guided FNAC. As a rule, FNAC is taken preoperatively of all salivary gland tumors (Atula et al. 1995 and 1996, Al-Khafaji et al. 1998, Filopoulos et al. 1998, Chhieng et al. 2000). Surgical biopsy should not be performed of the major salivary gland neoplasms, since there is a risk of facial paresis and tumor spread. If a diagnosis of a malignant tumor is made from the surgical specimen further imaging studies are performed postoperatively depending on the tumor type.

In addition to the histological diagnosis, the TNM classification is the only pertinent prognostic factor of SGC. The TNM classification system is based on tumor size (local extension of the tumor), metastasis to regional lymph nodes and distant metastases. The most recent changes to the TNM classification relate to a revision of the definition of T3 (Sobin and Wittekind 2002) and the division of T4-tumors which have been divided into T4a (moderately advanced local disease) and T4b (very advanced local disease), leading to the stratification of Stage IV into Stage IVA (moderately advanced local/regional disease), Stage IVB (very advanced local/regional disease) and Stage IVC (distant metastatic disease) (Edge 2010). Tumors arising in the minor salivary glands are classified according to the criteria for other carcinomas by anatomic site of origin (Sobin and Wittekind 2002).

2.11. Treatment

The principal goal for the treatment of SGC is disease control and preservation of nerve, vascular and muscle function and secretion of saliva. Despite thorough preoperative assessments, very often the diagnosis malignancy is not known prior to surgery.

Surgery is the main treatment of SGC. Due to anatomical limits of each site, the feasible margins vary to a large extent. In parotid gland tumors, removing the superficial lobe with an adequate margin is the recommended approach. Accurate identification and preservation of the facial nerve are crucial to successful surgery. The whole gland is removed if the tumor is located deep in lobe. Skin, muscles, nerves, mandible and temporal bone are resected, if tumor invasion is present (Mayers and Ferris 2007).

In the case of submandibular and sublingual gland tumors, the whole gland is removed, as well. Excision is appropriate of minor salivary gland tumors, as is the procedure for other malignancies at this site. Intraoperatively biopsies may be helpful for frozen-section evaluation to examine the surgical margins and lymph nodes and to guide the extent of surgery to ensure complete tumor resection; this holds true for all SGCs (Witt et al. 2004). An accurate histopathological classification of salivary gland neoplasms by frozen sections is difficult. If the SGC is in the submandibular gland, wide resection of adjacent soft tissues in the submandibular triangle may be needed, and this necessitates limited neck dissection (Eisele and Kleinberg 2004, Shah and Patel 2003).

Regarding surgery of adenoid cystic carcinomas, the extent of the resection should be performed with consideration of the presence of subclinical and perineural spread, which may occur far beyond the clinically palpable tumor mass (Eisele and Kleinberg 2004). This is a complicated matter, if the exact diagnosis is not known before surgery.

A large tumor and a high tumor grade are risk factors for neck lymph node metastasis. Neck dissection is performed of clinically metastatic nodes in all types of SGC. Modified radical neck dissection with preservation of the spinal accessory nerve, the jugular vein and sternocleidomastoideus muscle is usually preferred if metastasis is evident (Witt et al. 2004). If extracapsular extension of the tumor is present in the neck, the invaded structures are resected to achieve total tumor removal (Eisele and Kleinberg 2004). Selective neck dissection is considered if the patient has moderate risk, *i.e.*, a high-grade tumor without evidence of metastases or a large low-grade tumor.

Armstrong et al. (1992) studied 474 SGC patients. Tumors sized 4 cm or more were associated with a 20% risk of occult metastases compared with 4% for smaller tumors. High-grade SGC had a 49% risk compared with a 7% risk for intermediate-grade or low-grade SGC. Furthermore, submandibular gland cancer patients have more often occult metastases than patients with parotid gland cancer (Armstrong et al. 1992). Frankenthaler et al. (1993) found that in parotid gland malignancies associated with facial nerve paralysis the risk of metastases is 33%, while the overall risk of metastases for high-grade tumors is 18%.

There are no compelling data to support the benefit of elective neck dissection if there is no clinical evidence of tumor spread to the neck (Eisele and Kleinberg 2004). In general, neck dissection is indicated when the risk exceeds 20%. Disadvantages include functional disturbances at neck. Eisele and Kleinberg (2004) recommend adjuvant radiation therapy for patients at high risk. In these circumstances postoperative radiation therapy of the ipsilateral neck reduces the frequency of local recurrence, especially when the tumor is large (T3-4), there is metastatic spread to the neck lymph nodes (N1-3) or the tumor grade is high (MEC, carcinoma ex pleomorphic adenomas, adenocarcinomas and squamous cell carcinomas) (Borthne et al. 1986, Armstrong et al. 1990, Beal et al. 2003, Kian Ang and Garden 2006). Also, neck dissection is indicated if surgical resection has been incomplete or if there is a local recurrence (Wang 1997, Voutilainen et al. 2002, Robson 2002).

Radiation therapy is principally adjuvant, seldom curative and frequently palliative (Wang 1997). Postoperative radiation therapy may improve local tumor control, if the margins of the surgical resection have been narrow, if cancer cells have been detected in the resection line, if a facial nerve sparing procedure has been performed with close tumor margins or if the tumor is situated in the deep lobe of the parotid gland. In other words, postoperative radiation therapy is indicated in most conditions except low-grade stage I SGC (Eisele and Kleinberg 2004).

Elective radiation therapy of the neck is usually indicated, if postoperative irradiation of primary site is used. Most patients are candidates for postoperative radiation therapy

(Armstrong et al. 1992), the exceptions being patients with low-grade malignancies (low-grade mucoepidermoid carcinoma and acinar cell carcinoma) staged T1 or T2. If tumor spread is extensive, palliative radiation therapy is beneficial. Curative radiation therapy may be attempted for inoperable patients (Forastiere et al. 2008).

Chemotherapy is reserved for selected patients for palliation of unresectable or recurrent disease and for clinical trials (Eisele and Kleinberg 2004, Vaughan 2001, Mendenhall et al. 2005). Chemoradiation (cisplatin) is also an option, although there are no published trials of this approach (Forastiere et al. 2008). Various agents, *e.g.*, paclitaxel and combinations of paclitaxel with cisplatin, doxorubicin, cyclophosphamide, or carboplatin have been reported to have therapeutic benefit in small series of patients with SGC (Forastiere et al. 2008, Tanvetyaton et al. 2009).

The radiosensitivity of salivary gland cancer is low and the tumors are located close to critical organs. Particle beam therapy, *i.e.* hadron therapy, improves the dose distribution. Neutron/ion therapy is an established treatment of adenoid cystic carcinomas (Douglas et al. 1999, Jerezek-Fossa et al. 2006).

Radiation therapy affects the function of the salivary glands. Buus et al. (2006) reported that half of the parotid gland function was lost when the radiation dose is 30 Gy. At doses below 25 or 30 Gy, recovery is substantial and the function returns to pretreatment levels within two years after radiotherapy (Li et al. 2007). The intensity-modulated radiotherapy (IMRT) technique offers a possibility to spare the function of the major salivary glands not affected by the SGC (Saarilahti et al. 2006).

2.11.1. Follow-up

Locoregional and distant disease recurrence may occur more than 20 years after the completion of the definitive treatment (Healey et al. 1970, Lewis et al. 1991, Mendenhall et al. 2005). The 10-year and 15-year cumulative probabilities of late recurrence in patients who were disease free at 5 years were 13% and 18%, respectively (Chen et al. 2008). Patients with high-grade tumors and no locoregional recurrence had the same risk of distant metastases as patients with locoregional recurrence. The non-high-grade histological types of SGC were associated with a lower risk of distant metastases, although the risk remained elevated life-long (Bradley 2001). Thus, Bradley has suggested that all patients with SGC, regardless of histology, should be followed up and clinically assessed at least once every 12 months for life (Bradley 2001). The National Comprehensive Cancer Network (NCCN) proposed that a physical examination should be done every 6 to 12 months until 5 years after completion of the treatment (Bradley 2001, Forastiere et al. 2008). In a questionnaire of the British Association of Head and Neck Oncologists, 12% of the members followed the patients with SGC for longer than 5 years (Joshi et al. 2010).

3. AIMS OF THE STUDY

The purpose of the present population-based nationwide study was to characterize the salivary gland cancer (SGC) patients and tumors diagnosed in Finland in 1991-1996, to determine the incidence and histological distribution of SGC, to describe the treatment modalities used in Finland and to identify new prognostic factors of SGC based on 10 years of follow-up of patients with SGC in Finland.

The specific aims were:

1. to determine the incidence of SGC and to review the diagnoses of salivary gland malignancies in Finland during the years 1991-1996 (I).
2. to describe the treatment modalities used and to correlate patient survival with the patient's gender and age, tumor site, histological type and stage (I).
3. to estimate whether the extent of the expression of Ki-67 and p53 correlates with the clinical outcome of patients with SGC. For this, the application of volume-corrected mitotic index method (VCI) was used for determining the Ki-67 value (II).
4. to examine the applicability of the results of automated image analysis for assessing the expression of CD34-positive vessels to establish prognostic criteria in acinic cell carcinoma, adenoid cystic carcinoma and mucoepidermoid carcinoma (III, VI).
5. to verify the expression and to evaluate the prognostic significance of MMP-1, -7, -9 and -13 in patients with acinic cell carcinoma, mucoepidermoid carcinoma, adenoid cystic carcinoma and salivary duct carcinoma (IV, V).
6. to analyze the prognostic significance of MMP-1, -7, -9, -13, Ki-67 and HER-2 for patients with epithelial-myoepithelial carcinoma (VII).

4. PATIENTS, MATERIALS AND METHODS

4.1. Study population

The study population consists of the patients with SGC diagnosed and reported to the Finnish Cancer Registry (FCR) (Finnish Cancer Registry 2009) during the period 1991-1996. The FCR maintains a database of all patients with cancer in the population of Finland (population 5.1 million in 1996) and has published statistics on cancer incidence since 1953. Coverage is close to 100% (Teppo et al. 1994). The FCR also collects mortality follow-up data through information in death certificates.

For this study, the patient data and the follow-up information were collected from the patient records of the five University Hospitals in Finland which are responsible for the treatment of SGC in catchment areas. The survival data was received from the Population Register Centre and Statistics Finland. The TNM classification of the carcinomas followed the 1997 guidelines of the International Union against Cancer (Sobin and Wittekind 1997).

This study is part of a larger research project of head and neck cancer, for which the Ministry of Social Affairs and Health has granted permission, Dnro 15/08/94. The license permits the use of the patient data and clinical material for retrospective research. In the beginning the project, professor Erkki Virolainen gave permission to study SGCs in the area of the Turku University Central Hospital. The data for this project has been collected as part of the regular health care for the treatment of SGC and the personal details of the patients have been kept secret.

4.2. Re-evaluation

The original paraffin blocks of the patient samples were collected from the hospitals and private pathology laboratories. All malignant salivary gland tumors covering the period of study were included in this study.

The total number of new SGCs diagnosed over the period of six years in Finland was 332, *i.e.*, approximately 55 new cases per year. Lymphomas (n=46) and squamous cell carcinomas not originating from salivary glands were excluded. Thus, the total number of patients with new epithelial SGCs of different histological type was 286, *i.e.*, approximately 48 patients per year. The original histological specimens available (n=237) were collected and reclassified according to uniform criteria of the WHO classification of 1991 (Seifert 1991) by two pathologists experienced in salivary gland pathology, Dr. Ilmo Leivo and Dr. Pekka Klemi. In addition to hematoxylin-eosin staining, applicable immunohistochemical and histochemical staining was performed to verify the reclassified diagnoses, when needed (Table 3). Mucoepidermoid carcinomas

were classified as low-, intermediate- and high-grade tumors based on the grading model by Auclair et al. (1992).

In four cases (1.7%) the reclassified diagnosis was changed from a malignant tumor entity to a benign pleomorphic adenoma, and these cases were excluded from the final assessment and the epidemiological evaluations. The primary diagnoses had been mucoepidermoid carcinoma, adenocarcinoma, low-grade malignant myoepithelioma and basal cell adenocarcinoma. The original diagnosis was changed from one malignant entity to another in 51 cases (21.5%).

4.3. Immunohistochemistry

4.3.1. Immunohistochemistry, protocol for assessment of Ki-67 and p53

Immunohistochemistry was performed on deparaffinized, 5 µm-thick sections after antigen retrieval using microwave oven heating. A monoclonal Ki-67 antibody, clone MIB-1, and murine monoclonal antibody, antihuman p53 protein, clone DO-7, specific for a formalin-resistant epitope of the N-terminus of the human protein reacting with both wild and mutant types of the p53 protein were used. The staining was performed with a Tech Mate 500+ immunostaining machine (Dako, Copenhagen, Denmark). For immunohistochemical staining, a peroxidase/diaminobenzidine LSAB+ detection kit (DAKO, K5001) was used. After deparaffinization and inactivation of endogenous peroxidase activity and blocking of cross reactivity with commercially HP Block reagent, the sections were incubated for 1 hour at room temperature with the primary antibody diluted at 1:300 for p53 and 1:100 for Ki-67. The primary antibody was localized by subsequent incubation of biotinylated antiprimary antibody with an avidin-biotin complex conjugated to horseradish peroxidase and diaminobenzidine. The slides were washed three times with phosphate buffered saline after each incubation and counterstained with hematoxylin. Substituting the primary antibody with nonimmune mouse serum performed as negative controls.

The criteria of concordance of the staining pattern for p53 and Ki-67 were assessed using a consulting microscope (Leitz, Dialux 22, Viereich Germany) by Dr. Heikki Luukkaa and Dr. Pekka Klemi. The former, who was unaware of patients' clinical status, did the final evaluation. Discordant cases were discussed, and a consensus was reached.

4.3.2. Immunohistochemistry, protocol for assessment of angiogenesis

Lyophilized mouse monoclonal antibody to human endothelial cells (CD34), clone QBEnd/10, was purchased from Novocastra Laboratories Ltd. (United Kingdom) and used as instructed. The avidin-biotin modification of the peroxidase-antiperoxidase method was used for the immunohistochemical studies of histological sections. 5 µm-thick sections were cut, placed on slides coated with 3-triethoxysilylpropylamine

(Sigma, St. Louis, MO, USA) and fixed at 37° C over night. The slides were deparaffinized in xylene and graded series of ethanol, and boiled in Tris-EDTA (ethylene diamine tetra acetic acid tris) for 15 minutes. Endogenous peroxidase activity was quenched in a 3% H₂O₂ methanol solution for 30 minutes. Slides were then placed in H₂O and phosphate buffered saline (PBS) and then submitted to pre-treatment using 0.4% pepsin (Merck Darmstadt, Germany) at 37° C for 30 minutes. The slides were subsequently rinsed with H₂O and PBS and blocked with 20% fetal calf serum in PBS for 20 minutes. The specimens were exposed to the primary antibody (1:400 in PBS) for 1 hour at room temperature. Then the tissue samples were washed with H₂O, followed by biotinylated rabbit anti-mouse immunoglobulin and, finally, avidin-biotin complex (Dako). The peroxidase reaction was then performed using 3,3'diaminobenzidine (Dako REAL™ DAB+ Chromogen). The slides were counter-stained with hematoxylin for 15 seconds, dehydrated with increasing concentrations of ethanol and xylene and mounted.

4.3.3. Immunohistochemistry, protocol for assessment of MMPs

All immunohistochemical procedures were performed on formalin-fixed and paraffin-embedded tissue samples. 4 µm-thick sections were cut. Immunohistochemical stainings were performed using a BenchMark XT (Ventana) immunostaining machine and UltraView Universal DAB Detection kit (Ventana). Murine monoclonal antibodies Anti-MMP-1, Ab-1, Mouse mAb 41-1E5, Calbiochem dilution 1:500 for MMP-1, Anti-MMP-7, Ab-7, Mouse mAb ID2, Thermo Scientific dilution 1:10 for MMP-7, Anti-MMP-9, Ab-9, Mouse mAb 56-2A4, Fitzgerald dilution 1:50 for MMP-9 and Anti-MMP-13, Ab-3, Mouse mAb 181-14G11, Calbiochem dilution 1:5 for MMP-13 were used as primary antibodies. The pH at epitope retrieval of the antigens was 8.4 (CC1, Ventana).

4.4. Assessment of immunohistochemical results

4.4.1. Evaluation of Ki-67 expression

The densest area of Ki-67 positive cancer cells was chosen. The cells were counted and the percentage of the field containing cancer was estimated from five adjacent fields of view. The volume-corrected Ki-67 positive cells/square mm tumor tissue (VCI Ki-67) were counted using an application of the volume-corrected mitotic index method, as described (Haapasalo et al. 1989). In the present study, five fields were counted, at a magnification of 400, which corresponds to about 1 mm² of neoplastic tissue in the section.

The formula of the VMI:

$$\text{VMI} = k \left(\frac{\sum_{i=1}^n \text{MI}}{\sum_{i=1}^n \text{Vv}} \right)$$

where

n = number of microscope fields studied (5 in this study);

MI = number of mitotic figures in a microscope field from the area of highest neoplastic cellularity. During the measurement the microscope was focused only once;

Vv = volume fraction of neoplastic tissue (%) as estimated by the area fraction of neoplastic tissue in the microscope field. This was estimated subjectively; and

k = coefficient characterizing the microscope:

$$k = 100/\pi r^2$$

Where r (in mm) = radius of microscope field (0.28 mm in this study).

Ki-67 values were counted using a computer application created by Dr. Leo Paljärvi. The cutoff value was selected using the cross-tabulation method in survival.

4.4.2. Evaluation of p53 expression

For evaluation of p53 expression, the specimen was first assessed by several low-power fields: the area containing most positive staining of cancer cells in the field of view was chosen and the percentage of stained cells out of all cells was determined.

The cut-off value that best divided the patients with regard to the survival was selected using the cross-tabulation method for survival.

4.4.3. Evaluation of angiogenesis, morphometry and automated image analysis

All samples with sufficient material available for histopathology from paraffin blocks were included in the study (34 in study III and 55 in study VI). Ten consecutive microscope fields of view were measured from all tumors presenting with heterogeneous tumor tissue, and the values were automatically stored for statistical analysis. Each measurement consisted of 65 536 pixels approximating 320 μm \times 600 μm . The number of vessels and their properties were determined by computer-based image analysis of vessels expressing CD34 staining above a preset threshold level. Quantitative densitometry and image analysis were performed using the CAS 200 (Beckton-Dickinson, Leiden, The Netherlands) automated image analyzer and proprietary software Cell Measurement Program (Laitakari et al. 2001 and 2003). Individual vessel size, vessel shape (determined as the square of the perimeter divided by the vessel cross-sectional area) and staining intensity by CD34 were measured using quantitative densitometry and morphometry (Näyhä and Stenbäck 2007).

4.4.4. Evaluation of MMP expression

In total, 103 samples for MMP-1, -9 and -13 stainings, and 107 for MMP-7 stainings of the original 164 samples were available. The most common histological types were studied: adenoid cystic carcinoma (n=48; 47 for MMP-7), mucoepidermoid carcinoma (n=21; 23 for MMP-7), acinic cell carcinoma (n=22; 24 for MMP-7) and salivary duct carcinoma (n=12; 13 for MMP-7). Additional patients from Finland and six from Toronto, Canada were included in the study of the epithelial-myoepithelial carcinoma (VII).

The intensity of cytoplasmic staining, 0 (= no staining), 1, 2 and to 3 (= strong cytoplasmic staining), and the percentage of cells stained were recorded. Multiplication of staining intensity and percentage was used as an index describing the result, as well as intensity, and percentage separately. The method is semiquantitative.

To standardize the staining of the sections, a multibloc of normal salivary glands served as a control for the staining in each staining batches. A weak positive staining intensity was seen in the epithelial cells of the salivary ducts and in some of the serous acinar cells. Mucus secreting cells were negative for MMP.

In the normal salivary gland the average intensity and percentage of positive epithelial cells MMP-1, -7, -9 and for -13 were (1 and 100%), (1 and 100%), (1.3 and 100%) and (1 and 100%) respectively. For the acinar cells the figures for MMP-1, -7, -9 and -13 were (0.6 and 13%), (1 and 60%), (1 and 70%) and (1 and 59%) respectively. The criteria concordance of staining pattern was assessed using a consulting microscope (Leitz, Dialux 22, Viereich Germany) by Dr. Heikki Luukkaa and Dr. Pekka Klemi. The final evaluation was done by the former who was blinded to the patients' clinical status. Discordant cases were discussed, and after consensus the result was recorded.

4.5. Assessment of HER-2 immunohistochemical staining

The expression of HER-2 was studied by immunohistochemical staining in samples of epithelial-myoepithelial carcinoma and the results were related to the clinicopathological characteristics and 10-year survival of the patients. The patient cohort was international and encompassed 6 patients from Finland and 6 from Toronto, Canada.

4.5.1. Immunohistochemical staining

Immunohistochemical staining was performed on 4 µm-thick formalin-fixed and paraffin-embedded tissue samples using a BenchMark XT (Ventana) immunostaining device and UltraView Universal DAB Detection kit (Ventana). A mouse monoclonal antibody Anti-HER-2 (Ventana) 790-2991 clone 4B5, ready to use dilution, was used as the primary antibody. Epitope retrieval pH of the antigens was 8.4 (CC1, Ventana).

4.5.2. Evaluation of HER-2 staining

HER-2/neu expression was evaluated by the degree of staining of the cancer cell membranes and was scored from 0 to 3 (+++), as for breast cancer (Isola et al 2004).

4.5.3. Automated silver enhanced *in situ* hybridization of the HER-2-gene

Automated silver enhanced *in situ* hybridization (SISH) of consecutive slides from the same paraffin blocks which were used for MMP-staining were stained according to Ventana's protocols for the INFORM HER-2 DNA and chromosome 17 probes with the Benchmark XT immunostaining machine using the ultraView SISH Detection kit and the instructions of Ventana. Both probes were labelled with dinitrophenol (DNP) and optimally formulated for use with the ultraView SISH Detection Kit and accessory reagents on Ventana's Benchmark series of automated slide stainers. The silver precipitation was deposited in the nuclei and a single copy of the HER-2 gene was visualized as a black dot. The specimens were then counterstained with Ventana Hematoxylin II for assessment by light microscopy.

4.5.4. HER-2 scoring criteria

HER-2 gene amplification was interpreted and classified according to the criteria and instructions provided by the manufacturer Ventana (INFORM HER-2 DNA Probe CD Package insert 2007):

- Signal enumeration: A single copy was counted as 1, two copies as 2, small clusters (3-6 dots together) as 6, large clusters and more than 6 dots together as 12.
- A HER-2/Chr17 ratio of less than 3 was counted as negative for HER-2 gene amplification.
- A HER-2/Chr17 ratio of more than 3 in at least 10 % of the tumor cells was counted as positive for HER-2 gene amplification.

4.6. Statistical analyses

The follow-up time for overall survival was calculated from the date of diagnosis to the date of death or to the end of data collection which was December 31, 2001 (I-III) or December 31, 2006 (IV-VII). The follow-up time for disease-specific survival was calculated from the date of diagnosis to the date of death due to SGC, to the date of death due to other causes or to the end of data collection. Patients who died due to other causes than SGC were censored at the time of their death for the disease-specific survival analysis. Survival curves were plotted over time with the non-parametric Kaplan-Meier method and compared with the non-parametric log-rank test. Univariate and multivariate Cox proportional hazards regression was used to estimate the associations between p53, Ki-67, MMP-1, -7, -9, -13, age, gender, tumor location, histological type and TNM stage with the disease-specific and overall survival of the patients.

Hazard ratios and their 95% confidence intervals (95% CI) adjusted for T-stage, N-stage, histological type and stage of the disease were compared with survival. MMP-1, -7, -9 and -13 were divided into three categories by lower quartile, 2nd and 3rd quartile combined and upper quartile. The statistically significant explanatory variables in the univariate models were then included in the multivariate analysis. Relative survival was defined as the ratio of observed survival of the SGC patients divided by the expected survival of standard population in Finland stratified by age, sex and year. The age-standardized incidence rates were calculated using a reference standard population in Finland. Patients were divided into groups by 5-year (III) and 10-year (VI) survival and the tumor vessels were compared respectively for size, shape, staining intensity and vessel density. The association between tumor vessel size, shape and staining intensity and 5-year survival was analyzed using logistic regression with generalized estimation equations which takes into account the correlation between measurements determined from the same patients (Hosmer and Lemeshow 2000). In publications IV–VII the 10-year-survival was accounted for analyses. The new 10-year survival rates were recalculated for this thesis summary (Tables 6 and 7). In publication VII the differences in categorical variables between deceased and survived patients was analyzed with Fisher's exact test and the differences in continuous variables with Mann-Whitney's U-test. In all analyses, a two-sided p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed with the SAS statistical software (Versions 8.02 and 9.1, SAS Institute Inc., Cary, NC, USA).

5. RESULTS AND DISCUSSION

5.1. Salivary gland cancer in Finland, 1991-1996 (I)

5.1.1. Incidence and histological distribution

5.1.1.1. Results

The data on all SGC patients (n=286) diagnosed in Finland in 1991-1996 and reported to the Finnish Cancer Registry were retrieved. In the current study, the incidence was 0.9 cases/100 000 inhabitants/year. A histological re-evaluation and retrospective study was made of 237 SGC patients; this included incidence, histological type, location, treatment and outcome. The study population consisted of 125 (53%) males and 112 (47%) females. Their mean age was 59 years (males 61 years, females 58 years). The patients were followed-up for an average of 94 months (range 1–191 months). The most common tumor location was the parotid gland (n=152, 64%), followed by the minor salivary glands (n=46, 19%), the submandibular gland (n=38, 16%) and the sublingual gland (n=1, 0.4%). The most frequent histological types were adenoid cystic carcinoma (n=65, 27%), mucoepidermoid carcinoma (n=45, 19%) and acinic cell carcinoma (n=41, 17%). The distribution after reclassification of the histological diagnoses is shown in Table 4.

5.1.1.2. Discussion

According to the Finnish Cancer Registry, the incidence of SCG in 1991-2000 was about 1.0/100 000 inhabitants/year (1.1 for men and 0.9-1.0 for women). The reported incidence of malignant salivary gland tumors varies internationally from 0.4 to 2.6 cases/100 000 inhabitants/year (Parkin et al. 2002, Ries et al. 1994, Sun et al. 1999). In Finland the age-adjusted incidence rates during the years 1958-1998 ranged from 0.6 to 1.0/100 000 person-years, and at the end of the period the figure was 0.7 (Finnish Cancer Registry 2009). These incidences are close to what has been reported in Sweden for 1960-1989, 1.3 cases (1.4 for men and 1.2 for women) (Östman et al. 1997).

Table 4. Histological distribution of salivary gland cancers in Finland in 1991-1996, (modified from paper I).

Histological type	N	%	No. males	No. females
Adenoid cystic carcinoma	65	27.4	35	30
Mucoepidermoid carcinoma	45	19.0	22	23
Acinic cell carcinoma	41	17.3	24	17
Undifferentiated carcinoma	18	7.6	13	5
Salivary duct carcinoma	13	5.5	10	3
Adenocarcinoma	9	3.8	7	2
Polymorphous low-grade adenocarcinoma	8	3.4	4	4
Squamous cell carcinoma	8	3.4	6	2
Carcinoma in pleomorphic adenoma	8	3.4	5	3
Epithelial-myoeptithelial carcinoma	5	2.1	5	0
Basal cell carcinoma	3	1.3	1	2
Oncocytic carcinoma	3	1.3	2	1
Papillary cystadenocarcinoma	3	1.3	1	2
Malignant myoeptithelioma	3	1.3	1	2
Clear cell carcinoma	2	0.8	1	1
Unclassified carcinoma	2	0.8	2	0
Mucinous adenocarcinoma	1	0.4	0	1
Total	237	100	139	98

Among our patients the most frequent histological type was AdCC. The distribution by histological type of SGC in this study differs significantly from some other reports. The corresponding numbers, regarding the most frequent types, AdCC and MEC, have been reported in Canada, Denmark, Slovakia, Sweden and Germany (Fitzpatrick and Theriault 1986, Östman et al. 1997, Therkildsen et al. 1998, Satko et al. 2000, Wahlberg et al. 2002, Kokemueller et al. 2004b). In most studies, however, MEC is the most common histological type, *e.g.*, in USA (Spiro 1985 Hocwald et al. 2001 Bhattacharyya and Fried 2005) and Brazil (Bhattacharyya and Fried 2005). In our study the second and third most common histological types were MEC and AcCC. Satko et al. (2000) has published the similar distribution in Slovakia as we have observed in Finland, where AdCC, MEC and AcCC are the most frequent types in this order (Satko et al. 2000).

Usually adenocarcinoma is the third most frequent histological type (Spiro 1985, Hocwald et al. 2001, Kokemueller et al. 2004b, Bhattacharyya and Fried 2005, Östman et al. 1997, Wahlberg et al. 2002, Fitzpatrick & Theriault 1986) and seldom the fifth most common, as in the present study. It is remarkable that large studies where the histological distribution is available are mainly from North America, north Europe and the Netherlands.

The results of epidemiological studies need to be viewed with some criticism. The reasons for the variation in the occurrence of the histologic types are not understood presently, as the etiologies of these diseases remain unknown. Data is often restricted and limited to a certain site or only to major salivary gland tumors. The study population is often limited to a single center, which creates selection bias and there may be ethnic

differences. Full population-based nationwide studies are rare (Pinkston and Cole 1999, Barnes et al. 2005).

The great number of histopathological entities of SGC and the existence of overlapping growth patterns between various histological types makes the diagnosis of SGC a true challenge for pathologists. Among our patients the original diagnosis was changed from one malignant entity to another in 51 cases (22%). In four cases (1.7%) the reclassified diagnosis was changed from a malignant entity to a benign pleomorphic adenoma. The effect of changed diagnosis on survival was not studied. Also, the registration methods may vary and it is even possible that all patients with cancer are not registered or that SGC is classified under some larger anatomical structure of the head and neck, and the SGC diagnosis is not recorded as such.

Since the current study was performed a new WHO histological classification has been established (Barnes et al. 2005), where some criteria and diagnoses have again been changed. New entities in the latest version are the clear cell carcinoma not otherwise specified, the carcinosarcoma, the metastasizing pleomorphic adenoma, the large cell carcinoma, the lymphoepithelial carcinoma and the sialoblastoma (Barnes et al. 2005) (Table 1).

The re-evaluation of the histopathological diagnoses was based on the 1991 WHO classification of SGC (Seifert 1991). During the recent decades the histopathological entities of SGC and their diagnostic criteria have changed repeatedly. The great number of histopathological entities of SGC, the existence of overlapping growth patterns between various entities and the structural heterogeneity of individual tumors makes the diagnosis of SGC a great challenge for pathologists. In the present study, in 51 cases (21.5%) the original diagnosis was changed due to interpretation difficulties. This suggests that it would be beneficial to restrict the histopathological diagnosis of salivary gland tumors to highly specialized pathologists and centers.

This is the first large study in which the occurrence of different histological entities of SGC has been studied in Finland. It was based on data collected by the FCR; all SGC-patients from the years 1991-1996 were included. Re-evaluation of the histology was performed using the original histological slides. Fine needle aspiration cytology was not used in this study, since a FNAC-based diagnosis is not as reliable as a histological one (Atula et al. 1995). The site of the primary SGC is usually easy to determine, with the exception of the squamous cell carcinoma of the major salivary glands, which usually presents with metastatic disease from a primary site in the head and neck region. Thus, these cases were excluded from the study.

5.1.2. TNM-stage distribution

5.1.2.1. Results

The TNM-stage distribution was: stage I 138 (58%) patients, stage II 17 (7%) patients, stage III 14 (6%) patients and stage IV 59 (25%) patients. The stage of 9 patients was unknown.

Most patients were diagnosed with stage I disease: adenoid cystic carcinoma, n=37 (57%); mucoepidermoid carcinoma, n=32 (71%); and acinic cell carcinoma, n=32 (78%). The second largest group comprised those with stage IV disease (25%); only 17 patients had stage II and 14 stage III disease. In parotid, submandibular and minor salivary glands, the stage was most often I: 63%, 60% and 55%, respectively. At these sites, stage IV disease was found in 23%, 27% and 36% of cases, respectively.

5.1.2.2. Discussion

Kokemueller et al. (2004b) studied all SGCs and reported a distribution by TNM-stage as follows: stage I 50%, stage II 8%, stage III 4% and stage IV 37%. In a study of Hochwald et al. (2001) only parotid and submandibular gland cancers were included and the stage distribution was as follows: stage I 51%, stage II 17%, stage III 0% and stage IV 32%. In a study of a concise population (n=40) in Finland, the figures were: stage I 58%, stage II 8%, stage III 3% and stage IV 33% (Koivunen et al. 2002). Clearly, all these studies as well as the present one arrive at a similar TNM-distribution of SGC.

In the USA, the figures for stage I disease, in parotid, submandibular and minor salivary glands, were 43%, 41% and 27%, respectively (Pinkston and Cole 1999). These figures were significantly lower compared to our study. The high proportion of cases of stage I disease in the present study may be a reflection of the high quality of the healthcare system in Finland, which is very similar in all parts of the country and covers the whole population.

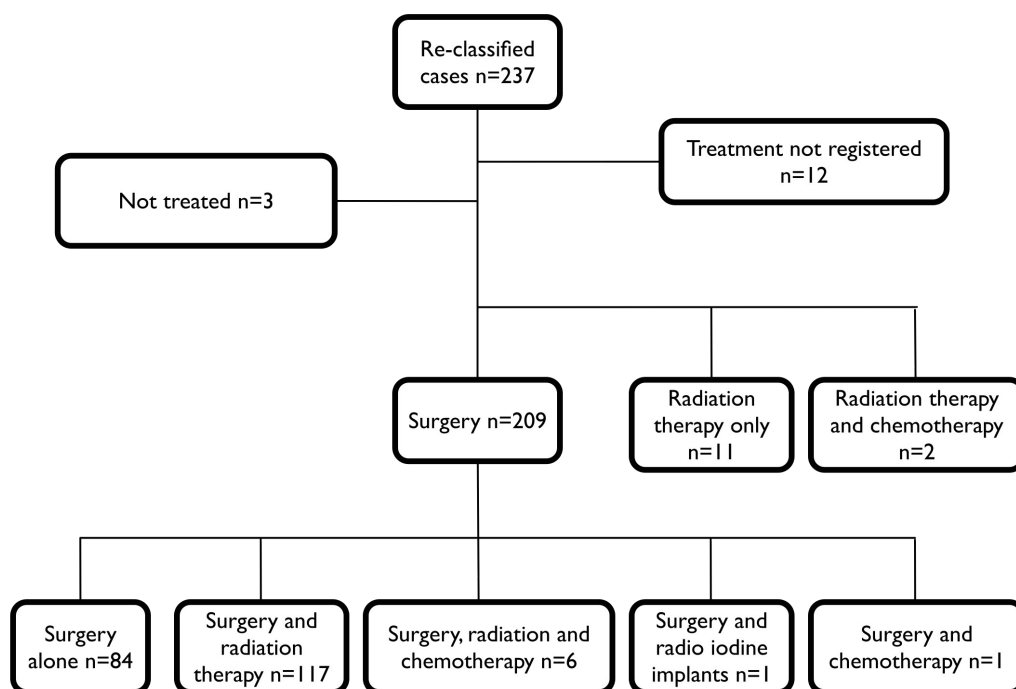


Figure 2. Schematic illustration of the treatment given to SGC patients, (modified from paper I).

5.1.3. Treatment

5.1.3.1. Results

In the study population of 237 patients the records of treatment were available of 225 patients (Figure 2). Surgery alone or in combination with other treatment modalities was used in 209 (88%) cases. Surgical treatment alone was performed on 84 (35%) patients. The neck was included in the surgical field in addition to the primary site in patients with evidence of metastatic disease. The exact numbers of this was not available due to registration methods.

Radiation therapy either alone or in combination with other treatment modalities was delivered to 136 (57%) patients.

The 5-year overall survival rate for patients who received only surgery was 70% and for patients who received combined therapy 48%, respectively.

Three patients were not treated with curative intent: in one case due to refusal, and in two cases due to a lack of co-operation or inoperable disease. Factors that influenced the choice of treatment were stage, histological classification, involvement of the facial nerve and radicality of the surgery.

Table 5. Treatment of salivary gland cancer in previous reports and in the present study.

Author	N	Population	Surgery alone	Surgery and radiotherapy	Radiotherapy alone
Therhault and Fitzpatrick 1986	271	Parotid gland cancer	25%	62%	13%
Therkildsen et al. 1998	251	All SGCs, Denmark	43%	49%	9%
Kokemueller et al. 2004b	155	All SGCs, Germany	86%	17%	n.d.
Pires et al. 2004	173	MEC all sites, Brazil	36%	21%	n.d.
Terhaard et al. 2004	565	All SGCs, Netherlands	20%	68%	n.d.
Present study	225	All SGCs, Finland	35%	88%	5%

n.d. not determined

5.1.3.2. Discussion

The treatment of SGC has varied remarkably in previous studies (Table 5). In our study 35% of the patients were treated only surgically, 5% only with radiotherapy and 49% with a combination of surgery and radiotherapy. In Denmark the distribution of treatment modalities are close to ours. Surgery is the mainstay of treatment, usually in about 80-90%

of patients (Table 5). Data on treatment was not always available in previous studies. The variation between studies may be explained by heterogeneous patient cohorts.

5.1.4. Histological type and survival

5.1.4.1. Results

The 10-year disease-specific survival rates for AdCC and AcCC were 60% and 90%. For all MEC the corresponding figure was 81%, for low-grade (n=29) MEC and for intermediate/high-grade group (n=13), the figures were 86% and 67%, respectively. The 5- and 10-year overall and relative survival rates are shown in Tables 6 and 7.

Table 6. The 5- and 10-year survival rates (%) of salivary gland cancer patients in Finland (n=237, years 1991-2006).

	5-year survival		10-year survival	
	Overall	Relative	Overall	Relative
Parotid gland	60	73	46	68
Minor salivary gland	59	80	41	67
Submandibular gland	39	51	34	47
Acinic cell carcinoma	83	96	76	90
Mucoepidermoid carcinoma	69	79	64	74
Adenoid cystic carcinoma	45	74	45	59
Stage I	78	94	70	89
Stage II	25	44	24	45
Stage III	21	43	21	30
Stage IV	23	31	17	21
All combined	62	71	50	65

Table 7. The 5- and 10-year disease-specific survival rates (%) of salivary gland cancer patients in Finland (n=237, years 1991-2006).

	5-year survival		10-year survival	
	Disease-specific	Relative	Disease-specific	Relative
Parotid gland	68	78	64	84
Minor salivary gland	80	91	71	96
Submandibular gland	54	62	50	65
Acinic cell carcinoma	93	103	90	108
Mucoepidermoid carcinoma	all	81	81	93
	low-grade	86	88	86
	intermediate	67	73	67
	and high-grade			
Adenoid cystic carcinoma	71	80	60	79
Stage I	89	100	85	110
Stage II	43	55	36	70
Stage III	43	52	43	62
Stage IV	30	35	22	27
All combined	68	78	64	83

5.1.4.2. Discussion

In AdCC 5- and 10-year overall survival rates of 72% and 50% have been reported by Kokemueller et al. (2002). 60% and 37% by Szanto et al (1984) and 73% and 45% by Spiro and Huvos (1992). In our study the 5-year overall survival was lower, 45%, but the 10-year figure, 45%, was similar to other studies. Spiro and Huvos (1992) reported also disease-specific figures for AdCC, which were 79% and 54%. They were close to our figures: 71% and 60%. In a study of Wahlberg et al. (2002), the relative 10-year survival of 74% was better than that in our series (59%).

In AcCC the relative 10-year survival in a study of Wahlberg et al. (2002), was 88%, which is close to our figure, 90%. In a study of Spiro et al. (1989) the 10-year overall survival was 85%, whereas our figure was 76%. Hoffman et al. (1999) reported a 5-year disease specific survival of 91% and overall survival of 83%; our figures were similar at 93% and 83%, respectively.

In MEC the relative 10-year survival in a study of Wahlberg et al. (2002) was 80%, whereas our figure was 93%. Evans (1984) reported 5-year overall survivals, for low and intermediate/high-grade MECs, which were 89% and 23%. For 10-year overall survival the figures were 79% and 23% (Evans 1984). Guzzo et al. (2002) found that 5-year disease free survival for patients with low and intermediate/high-grade MEC was 89%, 23% and the 10-year disease free figures were 79%, 23%, respectively. Our disease-specific figures for 5-year survival were 86%, 86% and for 10-year survival 67%, 67%, respectively.

5.1.5. *Site and survival*

5.1.5.1. Results

The outcome for different histological types and stages varies considerably on different sites. Of the three most common histological types, 80% of MEC and 88% of AcCC were in the parotid gland, whereas AdCCs were located on each site in practically even numbers: minor salivary gland 35%, parotid gland 32% and submandibular gland 32%. The 10-year disease-specific survival rate was more favorable for minor 71% (n=46) and parotid 64% (n=152) glands than for submandibular gland 50% (n=38). The 5- and 10-year overall, disease-specific and relative survival rates are shown in Tables 6 and 7.

5.1.5.2. Discussion

Vander Poorten (2002) studied minor, parotid and submandibular SGCs and reported the 5- and 10-year disease specific survivals for the minor salivary glands: 76% and 74%, for the parotid glands: 59% and 54% and for the submandibular glands 61% and 51%, respectively. These figures are close to ours. In a study of Wahlberg et al. (2002) the 10-year relative survival for parotid SGC was 72% and for submandibular SGC 65%. In parotid SGC our figure is 12% higher and in submandibular SGC similar.

5.1.6. Survival – general aspects

5.1.6.1. Results

The disease-specific 10-year survival rate was 64% for all SGCs and for stages I-IV it was 85%, 36%, 43% and 22% ($p < 0.001$; log-rank test) (Table 7). In the present study, stage I was the most important independent prognostic factor in Cox's disease-specific multivariate analysis ($p = 0.0002$; log-rank test) (Table 9). Stage IV disease was more frequent in males (31%) than females (21%). In contrast, stage I disease occurred more often in females (68%) than in males (54%).

5.1.6.2. Discussion

The 5- and 10-year relative survival rates in the USA (1988-2001) were 74% and 69% (SEER 2010). In a Danish study of Therkildsen et al. (1998) with 251 patients, the 5-, 10- and 15-year overall survival rates were 70%, 54% and 40% respectively, which are close to our 5- and 10-year figures, 62% and 50% respectively. In a Swedish series of 2465 patients with parotid and submandibular cancers, the 10-year relative survival rate was 72%. For the submandibular gland the survival improved in 1960–1989 from 54% (1960-1969) to 70% (1970-1979) and 65% (1980-1989) (Wahlberg et al. 2002). A study of 155 SGCs from Germany showed 5, 10 and 15 year overall survival rates of 66%, 48% and 40%, which were similar to ours (Kokemueller et al. 2002).

Table 8. Cox's multivariate analysis of prognostic factors of patients with salivary gland carcinomas with regard to 10-year disease-specific survival.

Variable	10-year disease-specific survival	
	P-value	Hazard ratio (95%CI)
Stage (II-IV vs. stage I)	<0.0001	7.73 (4.67-12.80)
Gender (Male vs. female)	<0.0001	2.516 (1.58-4.00)
Age ^a	0.0001	1.03 (1.01-1.04)
Histopathological diagnosis (Adenoid cystic carcinoma vs. others)	0.01	1.84 (1.14-2.97)

^aAnalyzed as a continuous variable.

Among females the prognosis was better than among males, which may be explained by TNM stage distribution. Doubts concerning the prognostic significance of the 1997 TNM classification (Sobin and Wittekind 1997) have been presented by several authors (Numata et al. 2000, Vander Poorten 2002). Numata et al. (2000) proposed that T1N1M0, T2N1M0, T3N1M0 and T4N0M0 should be classified as Stage III. Vander Poorten et al. (2002) studied patients with parotid carcinoma and arrived at the same conclusion as Numata et al. (2000), *i.e.*, that T3N1M0 and T4N0M0 should not be classified as Stage IV. Consequently new classifications have been published (Sobin and Wittekind 2002, Edge 2010). Thus, comparisons between different studies are even more difficult.

Varying histopathological examination methods, sampling patterns, methods of analysis, use of special stains and experience in performing analysis of findings also affect the results.

Survival varies significantly by histological type, stage and site. These variables assist in predicting the prognosis in the three largest histological groups. However, stage, Ki-67, lymph node status, gender and age were the most important prognostic factors in Cox's disease-specific multivariate analysis (Table 9). It is even more difficult to predict the outcome of patients with less common histological tumor types. New diagnostic and predictive tools are needed to determine the optimum treatment for each individual patient with SGC.

Table 9. Cox's multivariate analysis of prognostic factors for 10-year overall and disease-specific survival in salivary gland carcinomas.

Variable	Multivariate analysis Overall survival		Multivariate analysis Disease specific survival	
	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)
Age*	<0.0001	1.04 (1.03-1.06)	0.018	1.02 (1.00-1.04)
N (N1-N3 vs. N0)	0.008	1.95 (1.19-53.21)	0.006	2.17 (1.26-3.77)
Gender (Male vs. Female)	0.002	1.86 (1.26-2.73)	0.006	1.97 (1.22-3.18)
VCI Ki-67 (<20 vs. ≥20)	0.001	2.11 (1.39-3.21)	0.001	2.47 (1.44-4.23)
Stage (II-IV vs. I)	0.002	2.18 (1.33-3.56)	0.0002	3.33 (1.78-6.24)

*Age was analyzed as a continuous variable.

5.2. Morphological markers in salivary gland neoplasms

5.2.1. Cell proliferation in salivary gland carcinomas (II)

5.2.1.1. Results

The proliferative capacity using the volume-corrected index of Ki-67 nuclear antigen immunohistochemistry was evaluated in a series of paraffin embedded sections of 212 SGC-samples. A cutoff value of 20 was chosen. VCI Ki-67 negativity ≤ 20 was found in 99 patients and positivity >20 in 111 patients. The 5-year overall survival rates for these groups were 77% and 39%, respectively (log-rank test, $p < 0.0001$). The 5-year overall survival rates for the three largest histological groups (AdCC, MEC and AcCC) combined were 84% (n=83) for VCI Ki-67 ≤ 20 and 50% (n=50) for VCI Ki-67 >20 . The following statistically significant explanatory variables were identified in Cox's

univariate model: age, lymph node status, gender and stage ($p < 0.0001$). These variables were included in the multivariate analysis and it turned out that a high VCI Ki-67 score was associated with poor 5-year overall survival in all histological types ($p=0.011$) (Table 10). By univariate analysis patients with MEC had also a poor outcome at 5 years ($p=0.002$). Supplementary information was brought by age ($p=0.0002$), lymph node status ($p=0.001$), gender ($p=0.002$) and stage ($p=0.019$) in all histological types (Table 10). The result was similar for the disease specific 5-year survival and the 5-year overall survival. In low-grade mucoepidermoid carcinoma ($n=20$) a low level VCI Ki-67 predicted for favorable prognosis (80%). Cox's multivariate analysis of the 10-year disease-specific survival showed also that a high VCI Ki-67 predicted poor survival (Table 9). In the epithelial-myoepithelial carcinoma ($n=11$) group, the average VCI Ki-67 was 44: of the patients who died ($n=5$) the average was 76.5 and of the survivors ($n=6$) it was 23.2. A higher VCI Ki-67 ($p=0.034$) predicted worse disease-specific survival.

5.2.1.2. Discussion

Salivary gland carcinomas often consist of a heterogeneous tumor mass, a varying mass of neoplastic cells, supporting connective tissue, inflammation, degeneration of cells and tissue necrosis, which complicates the diagnostics and workup counts of a sample. The volume-corrected mitotic index (VMI) is a useful tool for this type of tumor entities (Haapasalo et al. 1989), and the present study is apparently the first one to report on the use of this technique to assess SGC. In this study salivary gland carcinomas were included and the VCI Ki-67 was a feasible prognosticator of survival. Previous studies of Ki-67 in SGC have reported similar findings. (Batsakis 1994, Hellquist et al. 1997, Nordgård et al. 1997, Xin and Paulino 2002, Lim et al. 2003, Skalova et al. 1994a, 1994b and 1996, Goode et al. 1998, Kiyoshima et al. 2001).

Table 10. Cox's univariate and multivariate analysis of prognostic factors of 5-year overall survival of patients with salivary gland carcinomas (modified from paper II).

Variable	Univariate analysis Overall survival		Multivariate analysis Overall survival	
	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)
Age*	<0.0001	1.04 (1.03-1.06)	0.0002	1.03 (1.02-1.05)
N (N1-N3 vs. N0)	<0.0001	5.29 (3.38-8.28)	0.001	2.73 (1.48-5.04)
Gender (Male vs. Female)	<0.0001	2.69 (1.69-4.29)	0.002	2.28 (1.37-3.82)
VCI Ki-67 (<20 vs. ≥20)	<0.0001	3.78 (2.36-6.04)	0.011	2.00 (1.17-3.41)
Stage (II-IV vs. I)	<0.0001	5.46 (3.38-8.83)	0.019	2.43 (1.16-5.11)

* Age was analyzed as a continuous variable.

The grading of mucoepidermoid carcinoma has been insufficient and difficult to perform (Seifert 1991, Ellis and Auclair 1996, Brandwein et al. 2001). In the present study mucoepidermoid carcinoma was low-grade in 22 patients. Of these, the VCI Ki-67 was low in 20 cases, and the 5-year overall survival of them was 80%. In the united group of intermediate (n=5) and high-grade (n=8) diseases the number of patients was 13. The 5-year overall survival of the low VCI Ki-67 patients (n=7) was 71% and in higher index (n=6) group 33%. Skalova et al. (1994a) reported a similar finding in which the high-grade MEC-patients presented high Ki-67 expression.

Brandwein et al. (2001) and Van Heerden et al. (2005) have determined that Ki-67 absence exists in 62% of mucoepidermoid cancer patients. A similar finding was also in our series: among the 38 MECs 24 (63%) had a VCI Ki-67 value <10.

Tumor heterogeneity is a well-known problem, especially in salivary gland cancer. Usually the highest proliferative area of the tumor will determine the clinical course; hence examining the tumor for the highest proliferative part may circumvent this problem (Jannink et al. 1996). This method does not require extensive experience or costly equipment and has achieved widespread acceptance in clinical practice and in studies on behavior of various tumors. The disadvantages include less than optimal reproducibility and sensitivity leading to variable results (van Diest et al. 1998). To compare results from one study to another, the exact area of high power field (HPF) must be defined since the area of field of vision can vary considerably by microscope configuration (van Diest et al. 1998).

5.2.2. Tumor suppressing protein p53 in salivary gland carcinomas (II)

5.2.2.1. Expression of p53 antigen in salivary gland carcinomas – Results

Expression of p53 tumor suppressor protein in salivary gland malignancies was evaluated by immunohistochemistry from paraffin embedded sections in a series of 212 SGCs. The cutoff value was 20%. p53 negativity ($\leq 20\%$) was found in 98 patients and positivity ($>20\%$) in 114 patients. The 5-year overall survival rates in these groups were 66% and 54%, respectively (p=0.046). The 5-year overall survival rates in the three largest histological groups (AdCC, MEC and AcCC) combined were 75% (n=63) for p53 ($\leq 20\%$) and 69% (n=71) for p53 ($>20\%$).

p53 expression did not provide additional value for the prediction of survival compared to the common clinical parameters: age, lymph node status, gender and stage. p53 expression did not associate with survival in multivariate Cox proportional hazards models (p=0.14).

5.2.2.2. p53 antigen in salivary gland carcinomas – Discussion

Overexpression on p53 has been associated with poor prognosis in several studies of SGC (Soini et al. 1992, Gallo et al. 1995, Papadaki et al. 1996, Zhu et al. 1997, Yamamoto

et al. 1998, Doi et al. 1999, Preisegger et al. 2001, Lim et al. 2003, Carlinfante et al. 2005, Jaehne et al. 2005, Ben-Izhak et al. 2009). On the other hand, overexpression of p53 has also been associated with a favorable prognosis in AdCC and in PLCC (Lazzaro and Cleveland 2000) and *e.g.* in laryngeal SCC (Hirvikoski et al. 1997). There are also studies on p53 according to which the value is doubtful in SGC (Kärjä et al. 1997, Kiyoshima et al. 2001, Xin and Paulino 2002).

The mechanism of p53 expression has been examined in numerous studies (Gold and Kim 2009) and the diversity of actions may explain the varying role in tumor growth and development. p53 is a tumor suppressor in its wild type while in the mutated form the suppression is reduced. Tumor suppression by p53 is mediated partly by the antiangiogenic activity of endostatin and tumstatin (Folkman 2006b, Teodoro et al. 2006). In angiogenic switch p53 is mutated and as a consequence the tumor cell proliferation increases relative to apoptosis. The levels of those endogenous angiogenesis inhibitors are decreased in the tumor and in its stroma. Furthermore the tumor switches to the angiogenic phenotype and a microscopic cancer becomes as a clinically detectable mass (Teodoro et al. 2006).

The association of p53 with both favorable and unfavorable outcomes of the patients and the reasons for the varying results reported are not clearly understood. Possible explanations include methodological problems. Fixation and preparation of tissue specimens are essential parts of immunohistochemical studies, which influence the results. Some antigens are preserved better in one fixative than in another (van der Loos 2007). The immunohistochemical technique of determining the p53 status has been criticized (Gallo et al. 1995). p53 expression is generally associated with occurrence of mutated p53, but the wild type p53 may also cause positive immunohistochemical staining.

The immunohistochemical analysis of the p53 protein product expression is a less specific than other methods of molecular analysis of the gene, such as single strand conformation polymorphism (Gallo et al. 1995). The background for p53 positivity is possible to proof using p53 mutation analysis (Nagao et al. 2002, Jaehne et al. 2005).

Interestingly, p53 is not only a diagnostic and prognostic marker, but it has also some therapeutical applications. p53 can induce cell cycle arrest and apoptosis under conditions of cellular stress of various kinds, *e.g.*, chemotherapeutics (Dodd and Slevin 2006). The key role of p53 inactivation for the development of cancer has generated an interest among drug developers to create compounds capable of restoring p53 function. Adenovirus -based p53 gene therapy is being studied preclinically in other carcinomas than SGCs (Di Cintio et al. 2010) and possibly some clinical applications may emerge also for the treatment of SGC in the future.

5.2.3. Angiogenesis in salivary gland carcinomas (III, VI)

5.2.3.1. Acinic cell carcinoma – Results

The role of angiogenesis as a prognostic criterion in acinic cell carcinoma (AcCC) was studied using computer-assisted automated image analysis. The study population consisted of 34 AcCC patients. Vessel numbers, vessel size, vessel shape and vessel wall antibody staining activity were measured. Altogether 10,385 vessels were stained for CD34 and were included in the study. The results are shown in Table 11.

5.2.3.2. Acinic cell carcinoma – Discussion

AcCC is generally regarded as a low-grade tumor, although also well differentiated tumors may behave unpredictably (Napier et al. 1995). The morphological characteristics of CD34 immunoreactivity were studied with computer-assisted analysis. Bigger vessel size, vessel irregularity and a lower intensity of CD34 positive vessel staining were related to an unfavorable prognosis. The two patients (TNM stage I and IV) who died of SGC had significant differences in vessel size, irregularity, staining intensity and density compared with patients who survived during the 5-year follow-up. For example, an irregular vessel pattern in the cancer tissue has been recognized previously and it may indicate aggressive tumor behavior (Näyhä and Stenbäck 2007, 2008). In hypoxia, or when tumor tissue produces pro-angiogenic growth factors, sprouting, bridging, and intussusceptive growth of existing blood vessels ensues (Folkman 1990, Risau 1997). In head and neck squamous cell carcinoma, the role of increased angiogenesis, which can be examined immunohistochemically with anti-CD34 monoclonal antibody, has been disputed, since results have varied from study to study (Erovcic et al. 2005, Ascani et al. 2005, Näyhä and Stenbäck 2008).

Table 11. Significance of angiogenesis in acinic cell carcinoma.

Vessel characteristic	Alive, mean value, n=32	Died of disease, mean value, n=2	Significance OR [odds ratio for change of standard deviation]	Conclusion
Size	272 μ m, SD 623	469 μ m, SD 1033	OR 1.16, 95% CI 1.02-1.31, p=0.024	Increased mean vessel size, patients died of disease
Irregularity	22.3, SD 22.1	28.3, SD 24.6	OR 1.19, 95% CI 1.10-1.29, p<0.001	Increased mean vessel irregularity, patients died of disease
Intensity	0.584, SD 0.119	0.555, SD 0.087	OR 0.75, 95% CI 0.58-0.96, p=0.024	Lower mean CD34 staining intensity, patients died of disease

5.2.3.3. Adenoid cystic carcinoma and mucoepidermoid carcinoma – Results

The study population consisted of 37 AdCC and 18 MEC patients. Vessel numbers, vessel size, vessel shape and vessel wall antibody staining activity were measured from vessels stained for CD34. Altogether 4433 vessels were measured from AdCC and 2615 from MEC. In AdCC 2151 and in MEC 500 vessels were measured from tumors of the patients who died of SGC (n=19 and 5) and 2282 and 2115 vessels from those who did not die of SGC (n=18 and 13) during the 10-year follow-up. The results are shown in Table 12.

The size of the vessels did not differ significantly from each other in either of the tumor types. In AdCC the shape factor was significantly higher in the tubular and cribriform types (28.02 ± 1.29) compared to the solid type (23.37 ± 1.55) ($p=0.023$), although this difference lost significance in the univariate disease specific survival analysis between the subtypes ($p=0.28$).

5.2.3.4. Adenoid cystic carcinoma and mucoepidermoid carcinoma – Discussion

The staining intensity of the vessels was significantly higher in MEC than in AdCC ($p=0.0005$). As expected, a difference was seen between different grades of MEC. A higher staining intensity was recorded in high-grade tumors of patients who died of the SGC (Table 12 A). In a previous study on MEC, increased mean vessel density correlated with a poor prognosis (Shi et al. 2007).

Thus, there were differences between the vessels of the aggressively growing tumors and indolent tumors (Table 12). The result of Nagy et al. (2003) may explain this by the angiogenic switch theory: tumor microvasculature changes in concert with the growth phase. Soares et al. (2007) studied the angiogenic switch and found that the vessel numbers increased (Folkman 2006a) during malignant transformation from pleomorphic adenoma to carcinoma ex-pleomorphic adenoma. In that study microvessel density measured with CD105 increased significantly during tumor progression, but for CD34 this was not seen (Soares et al. 2007).

In AdCC, the shape factor was significantly higher in the tubular and cribriform types (28.02 ± 1.29) than in the solid type (23.37 ± 1.55) ($p=0.023$), but when data was adjusted for disease specific survival, significance was lost. This was probably due to the low number of patients.

5.2.3.5. Morphometry – Discussion

The extent, structure and characteristics of angiogenesis is easily analyzed by visualizing the vessels in tissue sections by the endothelial cell-associated antibody CD34 (Tanigawa et al. 1996). Additional information can be obtained by using computer-assisted morphometry, which allows combining morphological and functional features (Laitakari et al. 2004, Näyhä and Stenbäck 2008). Comparisons of results within the same study

are more reproducible than with visual analysis or histoscore determinations (Barth et al. 1996, Risau 1997).

Table 12. Significance of angiogenesis in adenoid cystic carcinoma and mucoepidermoid carcinoma (model-adjusted mean±standard error), A Intensity, B Density.

A		Vessel characteristic	Died of disease	Alive or died of other causes	Detected difference	Conclusion
AdCC		Intensity			0.62 ± 0.01	Higher staining intensity in MEC than in AdCC, p=0.0005
MEC					0.68 ± 0.01	
MEC	Low grade	Intensity		0.71 ± 0.02		Did not die in disease: lower staining intensity in high grade, p=0.018
	High grade			0.63 ± 0.04		
	Low grade	Intensity	0.61 ± 0.05			Died in disease: higher intensity in high grade, p=0.018
	High grade		0.71 ± 0.04			
B		Vessel characteristic	Died of disease	Alive or died of other causes	Detected difference	Conclusion
MEC	Low grade	Density			161 ± 18 mm ²	Higher density in low-grade than in high grade, p=0.010
	High grade				93 ± 24 mm ²	
		Density	104 ± 24 mm ²	173 ± 15 mm ²		Higher mean vessel density, better survival, p=0.017

AdCC = adenoid cystic carcinoma, MEC = mucoepidermoid carcinoma

The method used in the present study has, however, certain limitations. The results are based on the expression of antibodies, the sensitivity, specificity and reliability of which are debatable (Laitakari et al. 2001, Laitakari et al. 2003, Laitakari et al. 2004, Risau 1997, Zhang et al. 2005). Clinical applications of this method are hampered by the need for rather expensive and cumbersome equipment and the lack of a standardized methodology (Laitakari et al. 2001, 2003, 2004, Näyhä and Stenbäck 2007, 2008). This study employed comparable staining methods with identical staining of controls and different groups as well as numerous controls. Vessel analysis, either using visual microscopic analysis or computerized methods, has its limitations as regards sensitivity, reproducibility and reliability (Risau 1997 Zhang et al. 2005). The area selected for measurement was also significant, since tumor classification as well as growth pattern are significant variables in this study. Due to the morphological pattern and mode of growth of the tumor, the vessels measured are located in the stroma immediately surrounding the tumor cell islets. Within the tumor islets the

vessel containing “hot spots” were too few, too small and too irregular for reliable and reproducible measurements.

Vessel staining characteristics were of prognostic significance. The computer program used in this study assigns every pixel a value on a level from 1 to 256, based on the intensity of the light absorbed by the stain. The values are absolute measurements and have a reproducibility and sensitivity exceeding 99% (Laitakari et al. 2001). The variability of the results as regards the measurement device is less than 0.01%. When the same stain and the same specimens are measured repetitively, the interobserver variability is less than 1% (Laitakari et al. 2001, Zhang et al. 2005). The problems of comparing different studies stem, for instance, from the fact that neoplasms are not homogeneous and selecting the area for study varies, histological slides contain only a small part of the tumor, selecting areas for the measurements are not universally agreed upon, staining methods are different and the results involve thousands of vessels and millions of pixels. Obviously, then, comparisons of different studies will produce disagreements. The problems could be solved to a large extent, were there only uniform agreement on which vessels should be selected for analysis. Differences in staining intensity may be related to vessel maturity: distinct staining may occur in mature, pre-existing vessels and weak staining in developing, neoplasm-associated, angiogenic, abnormal and less well-functioning vessels. Although logical, this concept is not universally accepted (Zhang et al. 2005).

5.2.4. Matrix metalloproteinases in salivary gland cancer (IV, V, VII)

5.2.4.1. Expression of MMP -1, -9 and -13 – Results

The index of MMP immunohistochemistry in the tumor tissue varied from 0 to 300. The mean value was 67.9 for MMP-1 (median 50), 107.8 for MMP-9 (median 100) and 124.1 for MMP-13 (median 100). High MMP-13 staining intensity showed a trend for poorer survival ($p=0.08$) among all patients. High MMP-13 staining intensity ($p=0.049$), a high percentage ($p=0.025$) and high index ($p=0.015$) were associated with poor survival among patients with acinic cell carcinoma. Among patients with salivary duct carcinoma, a high MMP-9 index ($n=12$) ($p=0.06$) and a high percentage ($p=0.05$) showed a trend for poorer disease-specific survival. High MMP-1 staining intensity ($p=0.06$) and a high index ($p=0.038$) had a trend for better overall survival among all patients. In AdCC a high percentage predicted better overall ($p=0.009$) and disease-specific ($p=0.017$) survival. The details of the immunohistochemical evaluation are shown in Table 13.

MMP expressions were further examined in patients with epithelial-myoepithelial carcinoma (EMC) (VII). Myoepithelial cells dominated the solid component of the tumor. The index of MMP immunohistochemistry in the tumor tissue varied from 0 to 300. Among EMC patients the strongest staining was observed for MMP-9 immunohistochemistry. The mean index for MMP-1 was 30 (median 15), for

MMP-9 88 (median 90) and for MMP-13 33 (median 35). A higher MMP-9 index predicted better overall survival ($p=0.033$). The detailed description of the immunohistochemical evaluation is found in paper VII.

Table 13. Results and conclusions of immunohistochemical expressions of MMP-1, MMP-9 and MMP-13 in salivary gland cancer, A Staining intensity, B %, C Index.

A	Histological type	Staining intensity	Conclusion
MMP-1	all	1 vs. 0; $p=0.10$, HR=0.62, 95% CI 0.35-1.09 2 vs. 0; $p=0.06$, HR=0.43, 95% CI 0.18-1.03 3 vs. 0; $p=0.28$, HR=0.51, 95% CI 0.15-1.72	Higher intensity, a trend for better overall survival
MMP-13	all	2 vs. 1; $p=0.69$, HR=1.12, 95% CI 0.65-1.92 3 vs. 1; $p=0.08$, HR=2.11, 95% CI 0.92-4.83	Higher intensity, a trend for poorer overall survival
	AcCC	2 vs. 1; $p=0.049$, HR=4.89, 95% CI 1.01-23.77	Higher intensity, poorer overall survival
B	Histological type	%	Conclusion
MMP-1	AdCC	2 vs 1; $p=0.009$, HR=0.31, 95% CI 0.13-0.74 3 vs 1; $p=0.50$, HR=0.75, 95% CI 0.33-1.70	Higher percentage, better overall survival
	AdCC	2 vs 1; $p=0.017$, HR=0.21, 95% CI 0.06-0.76 3 vs 1; $p=0.84$, HR=1.10, 95% CI 0.41-2.95	Higher percentage, better disease-specific survival
MMP-9	SDC	2 vs.1; $p=0.39$, HR=2.60, 95% CI 0.30-22.87 3 vs.1; $p=0.05$, HR=15.24, 95% CI 0.96-241.72	Higher percentage, a trend for poorer disease-specific survival
MMP-13	AcCC	2 vs.1; $p=0.025$, HR=6.22, 95% CI 1.25-30.80 3 vs.1; $p=0.39$, HR=2.18, 95% CI 0.36-13.17	Higher %, poorer overall survival
C	Histological type	Index	Conclusion
MMP-1	all	2 vs. 1; $p=0.038$, HR=0.53, 95% CI 0.29-0.96 3 vs. 1; $p=0.17$, HR=0.64, 95% CI 0.34-1.21	Higher index, better overall survival
MMP-9	SDC	2 vs. 1; $p=0.46$, HR 2.31, 95% CI 0.25-21.14 3 vs. 1; $p=0.06$, HR 12.81, 95% CI 0.93-176.17	Higher index, a trend for poorer disease-specific survival
MMP-13	AcCC	2 vs. 1; $p=0.85$, HR=1.16, 95% CI 0.26-5.17 3 vs. 1; $p=0.015$, HR=14.56, 95% CI 1.67-126.82	Higher index, poorer overall survival

AcCC = acinic cell carcinoma, AdCC = adenoid cystic carcinoma, MMP = matrix metalloproteinase, SDC = salivary duct carcinoma

5.2.4.2. Matrix metalloproteinases -1, -9 and -13 – Discussion

MMP-1, MMP-9 and MMP-13 have prognostic value in various human malignancies, as previously shown (Johansson et al. 1997, O-Charoerat et al. 2002, Luukkaa et al. 2006, Stokes et al. 2010). Our findings corroborate the results previously reported for HNSCC patients.

Surprisingly, high MMP-1 expression showed a trend for better survival in the whole patient population and in the group of patients with AdCC. In previous reports, elevated levels of MMP-1 in MEC and AdCC were found by EIA (enzyme immuno assay) from homogenates and by immunohistochemistry from AdCC samples (Kayano et al. 2004, Westernoff et al. 2005); these values resulted from comparisons with healthy salivary gland tissue. High MMP-1 expression has also been associated with a poor prognosis of patients with oral squamous cell carcinoma (Ala-aho and Kähäri 2005, Vihinen et al. 2005).

Of the histological types studied in MMP-9 expression was found a trend for poor survival only in SDC. High MMP-13 intensity had a trend level significance for poor overall survival. This association was strongest for AcCC of the histological types studied. Although both MMP-13 and MMP-1 can cleave fibrillar collagens, the substrate specificity of MMP-13 is markedly wider than that of MMP-1, and it can also effectively cleave components of basement membrane, as can MMP-9 (Ala-aho and Kähäri 2005). In the study of EMC, the average staining levels of MMP-1 and MMP-13 were similar, whereas the level of MMP-9 was higher. In the patients with a higher level of MMP-9 survival was better. In a study of Stokes et al. (2010), head and neck SCC-patients with lymph node metastases, the expression of MMP-9 was reduced. Thus, both MMP-13 and MMP-9 seem to be able to promote invasion of SGC cells by enabling them to cleave basement membrane, in contrast to MMP-1, which primarily cleaves type I collagen and not basement membrane components. It is also possible that these MMPs play a role at different stages of salivary gland carcinogenesis.

Until now, the connection between MMP and survival in SGC had not been studied. As reported in the literature, MMP-1, MMP-9 and MMP-13 are of prognostic value in various human malignancies including head and neck carcinoma (Riedel et al. 2000, Westernoff et al. 2005, Luukkaa et al 2006). Collagenases, MMP-1 and MMP-13 may promote malignant cell invasion and metastasis by initially cleaving triple helical fibrillar collagens of types I, II, III and V into fragments, which denature into gelatin, which is further degraded by other MMPs, such as gelatinases (MMP-9). Moreover, gelatinases degrade the components of basement membrane (Ala-aho and Kähäri 2005). The connection of metastasis formation and MMP expressions has not been studied in present work.

5.2.4.3. Expression of MMP -7 – Results

The index of MMP-7 staining by immunohistochemistry in the tumor tissue varied from 0 to 300. The mean value was 75.6 (median 60). In EMC the mean index for MMP-7 was 50 (median, 10). Detailed descriptions of the immunohistochemical evaluations are

found in papers V and VII and the main results are shown in Table 14. Lower intensity of MMP-7 was associated with the poorer prognosis of patients with both acinic cell and mucoepidermoid carcinoma. VCI Ki-67 results from a former study (II) were included for comparison and turned out to be an important prognostic factor for survival of the entire data set ($p < 0.0001$, HR 4.7, 95% CI 2.3-9.8, Cox's regression analysis). Higher T class predicted worse overall survival when the analysis was adjusted for age (2-4 vs. 1, mean survival time; 7.0 vs. 12.1 years; $p = 0.004$, HR 2.5, 95% CI 1.3-4.5). However the MMP-7 staining intensity, the percent of staining cells and the index did not associate with overall survival when the analysis was adjusted for age (Table 15).

Table 14. Significance of MMP-7 expression for patient survival.

Histological type	Staining intensity	Conclusion
AcCC	0-1 vs 2-3; $p=0.047$, HR 6.5, 95% CI 1.0-41.7, age adjusted analysis.	Low intensity, poorer survival
	0-1 vs 2-3; $p=0.047$, HR 13.8, 95% CI 1.0-182.7, age-adjusted disease-specific analysis	
MEC	0-1 vs 2-3; $p=0.010$, HR 9.3, 95% CI 1.7-50.0, age adjusted analysis	Low intensity, poorer survival

AcCC = acinic cell carcinoma, MEC = mucoepidermoid carcinoma

Table 15. Age-adjusted Cox regression analysis of MMP-7 immunohistochemistry and overall survival of patients with salivary gland carcinoma.

Variable	Age-adjusted analysis (n=107)	
	P-value	Hazard ratio (95% CI)
MMP-7 staining intensity (2-3 vs. 0-1)	0.15	0.69 (0.42-1.14)
MMP-7 percent of staining cells	0.36*	
2 vs. 1	0.19	1.49 (0.82-2.69)
3 vs. 1	0.83	1.08 (0.52-2.25)
MMP-7 index	0.19*	
2 vs. 1	0.08	1.74 (0.93-3.28)
3 vs. 1	0.60	1.22 (0.58-2.57)

* Overall p-value

5.2.4.4. Matrix metalloproteinase -7 – Discussion

Interestingly, high rather than low MMP-7 expression was associated with better survival of patients with acinic cell and mucoepidermoid carcinoma. To our knowledge, neither the expression of MMP-7 nor its association with survival of patients with SGC has been studied before. In previous studies involving patients with other solid tumors, e.g., pancreatic adenocarcinoma (Yamamoto et al. 2001) and esophageal squamous cell carcinoma (Yamashita et al. 2000), increased MMP-7 expression in cancer cells has been

associated with a poor prognosis. The expression of MMP-7 has also been associated with recurrence of hepatocellular cancer (Yamamoto et al. 1999) and with lymph node metastasis of gastric carcinoma (Yamashita et al. 1998). An inverse association between MMP-7 expression and tumor size, N positivity, large pT and poor differentiation has been reported for papillary thyroid carcinoma (Ito et al. 2006). In conclusion, in our study, lower staining intensity of MMP-7 was associated with poor overall survival of patients with acinic cell and mucoepidermoid carcinomas.

The reduced expression of MMP-7 in cancer cells may be caused by incomplete differentiation, since healthy salivary gland tissue also expresses MMP-7 (Saarialho-Kere et al. 1995). This observation is not surprising, since certain MMPs are tumor suppressive (Lopez-Otin and Matrisian 2007).

MMP-7 is not expressed in healthy human skin keratinocytes, but is abundant in Bowen's disease and cutaneous squamous cell carcinoma (SCC); it is most abundant in poorly differentiated SCC. Thus, the expression of MMP-7 increases with the degree of malignancy (Kivisaari et al. 2008). In the present study the expression of MMP-7 was weak in normal salivary gland tissue, as reported earlier by Saarialho-Kere et al. (1995), but stronger in SGC. This conforms with the concept that malignant transformation in salivary gland tissue causes a similar change in the expression of MMP-7 as in skin cancer. Specific induction of MMP-7 expression in malignant salivary gland tissue may promote aggressive biological behavior of SGC; MMP-7 could thus act as an early marker of SGC. This hypothesis raises the interesting prospect of screening and early detection of SGC by assessing MMP-7 in the saliva.

Several factors are involved in tumorigenesis and, of course, the MMPs do not operate in isolation. In murine mammary gland cell lines MMP-7 processes other growth factors into soluble forms; MMP-7 activates, through proteolysis, other proteinases which, in turn, promote cell proliferation. MMP-7 mediates the processing of ErbB4 involved in upstream MMP-7 activity in mammary gland tumorigenesis (Lynch et al. 2007). Mammary epithelial cells transiently exposed to MMP-7 undergo enhanced apoptosis, while cells that are subjected to continuous exposure and constant circulating levels of Fas ligand (FasL) show a 50% reduction in Fas-induced apoptosis (Fingleton et al. 2001, Vargo-Gogola et al. 2002).

5.2.4.5. Matrix metalloproteinases – Discussion

It is known that the quantity of proteolytically active enzyme capable of degrading the stromal matrix is determined by the molar balance between the non-specific inhibitors in the serum (*e.g.*, α 1-proteinase inhibitor and α 2-macroglobulin) and the active inhibitors of metalloproteinases in the cells and tissue. Similarly, the membrane-anchored regulator of matrix metalloproteinases (RECK) inhibits tumor invasion and angiogenesis (Oh et al. 2001).

The prevailing view is that proteases promote tumor progression and metastasis. This view has led to the development of small-molecule inhibitors for the treatment of cancer. Examples include molecules targeting MMPs and plasminogen activators. In clinical trials these molecules have, however, had no effect on the malignant tumors and, in some patients treated with broad-range metalloproteinase inhibitors, there may even have been an acceleration of tumor growth. This finding suggests that some proteases, *e.g.*, MMP-9 acting as an angiogenesis inhibitor, might play an anti-tumorigenic role (Lopez-Otin and Matrisian 2007, Martin and Matrisian 2007).

Immunohistochemistry allows for immediate assessment of the expression intensity and extent in cell pattern. This facilitates prediction of the biological relevance of the immunohistochemical findings. MMP are expressed in cancer tissue and, to some extent, in the peritumoral area, preferentially in fibroblasts and inflammatory cells (Ala-Aho and Kähäri 2005, Vihinen et al. 2005).

Multiple factors influence the role and significance of matrilysins, collagenases and gelatinases in the pathogenesis, growth, invasion and metastasis of cancer. The general hypothesis is that overexpression of a certain MMP, either by tumor cells or by the surrounding stroma, leads to malignant transformation. Perhaps the expression of certain MMPs, either at the primary or the metastatic site, promotes multiple stages of cancer progression by providing a pro-tumorigenic and protective milieu in the tissue (Martin and Matrisian 2007).

In the current study lower MMP-7 staining intensity was associated with worse overall survival of patients with acinic cell and mucoepidermoid carcinoma, and MMP-13 had a trend level significance for poor prognosis of patients with salivary gland cancer, suggesting that they may be useful prognostic factors in patients with SGC.

5.2.5. HER-2 immunohistochemistry and *in situ* hybridization (VII)

5.2.5.1. Results

The expression of HER-2 in epithelial-myoeepithelial salivary gland carcinoma was studied by immunohistochemistry and HER-2 gene amplification by silver enhanced *in situ* hybridization in a series of 12 paraffin-embedded histopathologic samples of patients from Canada and Finland. In one patient, HER-2 oncogene amplification was observed.

5.2.5.2. Discussion

Salivary duct carcinoma (SDC) shares significant morphologic and immunophenotypic similarities with ductal carcinoma of the breast, including HER-2/neu (c-ErbB-2) overexpression. In SGC, the expression of HER-2 is most frequently seen particularly in SDC (Skalova et al. 2003, Laurie and Licitra 2006, Nabili et al. 2007, Williams et al. 2007, Johnson et al. 2008). In some reports HER-2 positivity has also been connected

to other histological types of salivary gland cancer, *e.g.*, to the high-grade epithelial phenotype of non-invasive carcinoma ex pleomorphic adenoma (Di Palma 2005, Matsubayashi and Yoshihara 2007), adenocarcinoma and to mucoepidermoid carcinoma (Laurie and Licitra 2006).

In adenoid cystic carcinoma, overexpression of HER-2 has been identified (Kärjä et al. 1994, Shintani et al. 1995, Gibbons et al. 2001), but also lower expression (4-58%) has been reported (Kernohan et al. 1991, Cho et al. 1999, Dori et al. 2002, Glisson et al. 2004), as has found complete absence of expression (Shrestha et al. 1992, Sugano et al. 1992, Dodd et al. 2006). In tubular and cribriform subtypes HER-2 has been more common than in the solid type (Shintani et al. 1995). Cho et al. (1999) reported that overexpression HER-2 in adenoid cystic carcinoma was associated with shorter disease free survival.

Some studies show that HER-2 is associated with aggressiveness of tumor types, *e.g.*, in carcinoma of pleomorphic adenoma (Sugano et al. 1992) and in mucoepidermoid carcinoma (Nguyen et al. 2003). Press et al. (1994) studied 58 MECs: 11 were HER-2 positive and correlated with poor survival.

In a phase II trials of lapatinib in HER-2 positive patients, the stabilization of disease for more than 6 months in AdCC and non-AdCC patients has been reported (Agulnik et al. 2007) and better outcome of non-AdCC patients (Vidal et al. 2009). There are two ongoing clinical trials of trastuzumab for treating patients with advanced salivary gland cancer (www.clinicaltrials.gov accessed March 24th2010). Moreover, development of HER-2 peptide based tumor vaccine has advanced to clinical trials (Anderson et al. 2009a).

The expression of HER-2 in epithelial-myoepithelial carcinoma has also been studied by others. Cho et al. (1995) studied the tumors from 26 EMC patients, which all were negative for HER-2 immunostaining. In our series of 12 patients, HER-2 gene amplification was positive in one patient who was alive and well after almost 14 years of follow-up. The number of patients was low due to a rare disease, and the prospects for statistical conclusions are limited.

Present knowledge and experience thus implies that HER-2 evaluation is applicable only to SDC. Therefore, HER-2 does not offer additional benefit as a prognostic factor in EMC.

5.2.6. Aspects of morphological methods

Salivary gland cancer is rare and the tumors are histologically diverse. Small numbers pose a big challenge for research.

Nevertheless, new potential prognostic factors were examined in the present study and detailed morphological and immunohistochemical analyses of tissue samples did

turn out to provide additional information, which is useful for the clinical setting. The findings of this study need to be applied with care and with a full understanding of the methodology, its specific pitfalls and limitations. Quantitative and specific biochemical and molecular biology methods are easier to apply and more practical, as well (Ohyama et al. 2002), but they are also sensitive to interpretations. Laser capture microdissection of tumor specimens, a method thus far not used in SGC, improves the methodological sensitivity and specificity by restricting the study to a targeted tumor cell population (Ohyama et al. 2002).

6. CONCLUSIONS

The tumors and data of patients with salivary gland cancer (SGC) diagnosed in Finland in 1991-1996 and reported to the Finnish Cancer Registry were studied. The total number was 237. The incidence of SGC, the histological distribution and treatment modalities were determined. New prognostic factors for salivary gland cancer were generated and tested; the follow-up time was 10 years for each patient. The following conclusions were drawn:

1. The incidence and results of the re-evaluation of the histology of the tumors corresponded to the results of former studies. The most frequent histological types were adenoid cystic carcinoma (27%), mucoepidermoid carcinoma (19%) and acinic cell carcinoma (17%), the 10-year disease-specific survival was 60%, 81% and 90%, respectively, and for all SGC 64%.
2. The outcome of patients was associated with treatment and histological type of the tumors, tumors stage, as well as patients' gender, and age. Surgery alone and in combination with other treatment modalities was used in 209 (93%) cases. Radiation therapy was given to 134 (57%) patients, of which 11 (5%) without surgery. By Cox's disease-specific 10-year multivariate analysis, stage I, male gender, age and histological type were the most powerful predictors of patient outcome.
3. The volume-corrected index (VCI) Ki-67 was a useful prognostic factor for patient survival. p53 expression did not provide additional information for prediction of survival.
4. Vessel number, vessel shape, vessel size and the intensity of CD34 positive staining were related to patient survival, but were not independent prognosticators of patient survival. Altered vessel characteristics and increased vessel number indicated an unfavorable prognosis for patients with mucoepidermoid carcinoma, and an association with survival was also found for patients with acinic cell carcinoma.
5. High expression of matrix metalloproteinases (MMPs)-9 and -13 had a trend level significance for poorer prognosis and MMP-1 showed a trend for better prognosis among patients with salivary gland cancer. Lower MMP-7 staining intensity was associated with worse overall survival in acinic cell and in mucoepidermoid carcinoma, when adjusted for age.
6. The staining pattern of MMP-1, -7, -9, -13 and HER-2 in epithelial-myoepithelial carcinoma was characterized. In this histological tumor type a high volume-corrected index (VCI) of Ki-67 was related to increased mortality.
7. The results showed that information can be obtained by using additional morphological and biological markers. Before large scale applications of these results to clinical practice, further studies with larger patient populations are needed.

7. SUMMARY

Salivary gland cancer (SGC) is a rare cancer. The tumors are clinically and morphologically a diverse group of neoplasms, which makes this cancer type a challenge for diagnosis and treatment.

The worldwide annual incidence varies from 0.4 to 6.5 patients per 100 000 person-years. In Finland the age-adjusted incidence rate has increased within 50 years from 0.5 to 1.5 per 100 000 person-years. Salivary gland tumors occur most often in the parotid gland, but the proportion of malignant tumors is highest in the minor and submandibular glands.

The histopathological classification is complex; the biological behavior of these tumors varies from benign to high-grade and often fatal malignancies. The morphological heterogeneity of SGC is well-known and presents a vast challenge for the diagnosis and classification of salivary gland tumors. This variation is present among different tumors as well as within the one and the same tumor mass.

Treatment of SGC is demanding. The mainstay of treatment is surgery combined with adjunctive radiation therapy, when required. Many of these tumors behave in an indolent fashion; however, late recurrences after 15-20 years are not rare. This impairs the ability of the clinician to draw conclusion about the efficacy of treatment. New prognostic tools are clearly needed to identify better patients at high risk of recurrence and death.

The present study encompassed a full, population-based nationwide cohort of salivary gland cancer patients and tumors diagnosed in Finland in 1991-1996. The purpose was to characterize the material, determine the incidence of SGC and the histological distribution, to describe treatment modalities used, and to generate new prognostic variables for salivary gland cancer. The follow-up time was 10 years.

The incidence of SGC among the whole population of Finland was, on average, 47.7 cases per year. After histological evaluation and re-evaluation of 237 cases the most frequent histological types were adenoid cystic carcinoma (n=65, 27%), mucoepidermoid carcinoma (n=45, 19%) and acinic cell carcinoma (n=41, 17%).

Surgery alone and in combination with other treatment modalities was most frequently used (n=209, 88%). Radiation therapy was given to 136 (57%) patients of which 13 (5%) without surgery.

The 10-year disease-specific survival of patients with stage I-IV disease was 85%, 36%, 43% and 22%, respectively, and of all SGC patients 64%.

The highest relative 10-year overall survival rate was recorded among patients with acinic cell carcinoma (90%), followed by mucoepidermoid (74%) and adenoid cystic carcinoma (59%). The corresponding disease-specific 10-year survival figures were

90%, 81% and 60%. Of the clinical indicators, advanced stage, male gender, advanced age and histological type were the most powerful predictors of patient outcome.

Cell proliferation as indicated by expression of Ki-67 was a useful prognostic factor for survival. A high volume-corrected index of Ki-67 (VCI Ki-67) was associated with worse survival of SGC patients, regardless of histological type ($p=0.011$) according to Cox's multivariate analysis and in mucoepidermoid carcinoma according to univariate analysis ($p=0.002$). A high VCI Ki-67 predicted poor survival ($p=0.001$) according to a 10-year disease-specific analysis. p53 expression did not provide information for prediction of survival ($p=0.14$) over and above what information the common clinical parameters contained.

Certain morphological characteristics were identified by computer-assisted analysis of CD34 immunoreactivity of vessels in acinic cell carcinoma, mucoepidermoid carcinoma and adenoid cystic carcinoma. In AcCC, bigger vessel size, vessel irregularity, and lower intensity of CD34-positive vessel staining may indicate an unfavorable prognosis. The staining intensity of CD34 positive vessels in MEC was higher than in AdCC. In MEC, poor survival was associated with higher staining intensity of intermediate and high-grade tumors and lower vessel density. In our experience, computer-assisted analysis of CD34 stained microvessels is a feasible complement to biochemical techniques, when a heterogenous histological cell pattern is presented.

A high level of MMP-13 had a trend level significance for poorer prognosis among patients with SGC and a high level of MMP-9 showed a trend for poorer prognosis for patients with salivary duct carcinoma. In contrast, a high level of MMP-1 had a trend for better prognosis for patients with salivary gland cancer. A low level of MMP-7 was associated with a poor prognosis for patients with acinic cell and mucoepidermoid carcinoma. Thus, these MMPs contribute to the progression and invasion of SGC.

The staining pattern of MMP-1, -7, -9, -13 and HER-2 in epithelial-myoepithelial carcinoma was characterized. A high VCI of Ki-67 was observed in patients subsequently deceased.

In conclusion, the incidence, the histological proportions of different histological types, treatment and a 10-year survival of patients with salivary gland cancer in Finland were determined. These results were comparable with previous studies referred to in the international literature. VCI Ki-67 correlated with to a reduced survival in SGC. Results of computer-assisted morphometric analyses of CD34 positive vessels indicate an unfavorable prognosis for patients with MEC and, to a lesser extent, with AcCC. The degree of expression of matrix metalloproteinases (MMPs)-1, -9 and -13 was associated with the prognosis of patients with salivary gland cancer. Expression of MMP-7 was associated with the prognosis of patients with acinic cell and in mucoepidermoid carcinoma. Characteristics of salivary gland cancer and histological types in Finland are presented.

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A handwritten signature in black ink, appearing to read 'Heikki Luukkaa', written in a cursive style.

Heikki Luukkaa

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