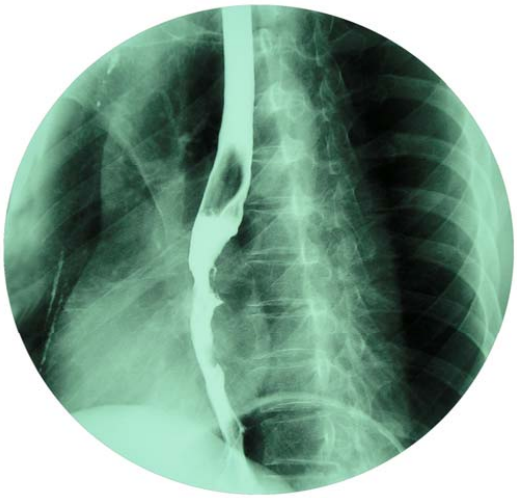
Clinicopathological significance of seprase, dipeptidyl peptidase IV and urokinase-type plasminogen activator in esophageal carcinomas



Mariusz Adam Goscinski

Thesis

Faculty Division the Norwegian Radium Hospital, Rikshospitalet HF,
Faculty of Medicine

UNIVERSITY OF OSLO

Department of Surgical Oncology
and Department of Pathology
The Norwegian Radium Hospital,
Rikshospitalet HF, Oslo, Norway

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To
Alexandra, Zofia, Anna, Jan and Stanislaw

TABLE OF CONTENTS

1. PREFACE

Acknowledgements

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Oslo, October 2008

Mariusz Adam Goscinski

Abbreviations

ACS	American Cancer Society
ADAM	Disintegrin And Metalloprotease Family
AJCC	American Joint Committee of Cancer
Bcl-2	B-cell CLL/lymphoma 2
Bcl-X	Anti-apoptotic Gene
b-FGF	Basic Fibroblast Growth Factor
BMI	Body Mass Index
BMP-6	Bone Morphogenetic Protein 6
c-Myc	Proto-oncogene Retrovirus-associated DNA Sequences
Cox-2	Cyclooxygenase 2
DAB-2	Disabled Homolog 2, Mitogen-responsive phosphoprotein
DPPIV	Dipeptidyl Peptidase IV
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMR	Endoscopic Mucosal Resection
ERCC3	Excision Repair Cross-complementing Rodent Repair Deficiency
FAP-α	Fibroblast Activation Protein-α
FAS-L	Fas Ligand (TNF superfamily, member 6)
GERD	Gastroesophageal Reflux Disease
GUS	Beta-Glucuronidase
HER2	Human Epidermal Growth Factor Receptor 2
HET1A	Immortalized Esophageal Keratinocytes (Cell Line)
HPV	Human Papillomavirus
hTERT	Human Telomerase Reverse Transcriptase
HUVEC	Human Umbilical Vein Endothelial Cells
iNOS	Inductible Nitric Oxide Synthase
INT-2	Fibroblast Growth Factor 3
KYSE	Human Japanese Esophagus Carcinoma Squamous Cell (Cell Line)
MMP	Matrix Metalloproteinase
MT-MMP	Membrane-type Matrix Metalloproteinase
Nd:YAG	Neodymium-doped Yttrium Aluminium Garnet
NFκB	Nuclear Factor Kappa B
NSAID	Non-steroidal Anti-inflammatory Drug
P16	Tumor Suppressor Gene
p16MST1	Tumor Suppressor Gene
PCR	Polymerase Chain Reaction
PDT	Photodynamic Therapy
PVDF	Polyvinilydene Fluoride
QRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RARβ	Retinoic Acid Receptor Beta
RFA	Radiofrequency Ablation
RT-PCR	Real-time Polymerase Chain Reaction
SCC	Squamous Cell Carcinoma

SIM	Specialized Intestinal Metaplasia
SIMP	Serine-type Integral Membrane Peptidase Family
TBS	Tris Buffered Saline
TGF-α	Transforming Growth Factor Alpha
TGF-β	Transforming Growth Factor Beta
TGH	Triiodothyronine, Glucagon and Heparin Mixture
TNM	Classification of Malignant Tumors
TP	Thymidine Phosphorylase Gene
TRAIL	Tumor Necrosis Factor-related Apoptosis-inducing Ligand
TRIS	Tris(hydroxymethyl)aminomethane
UICC	International Union Against Cancer
uPA	Urokinase-type Plasminogen Activator
uPAR	Urokinase-type Plasminogen Activator Receptor
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

TNM Classification / Staging

Histological classification of carcinomas according to UICC Global Cancer Control, TNM Atlas (UICC 2005).

Anatomical subsites of the esophagus:
- Cervical esophagus
- Intrathoracic esophagus
  1. Upper thoracic portion
  2. Mid-thoracic portion
  3. Lower thoracic portion

T – Primary Tumor

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor invades lamina propria or submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades adventitia
T4	Tumor invades adjacent structures

N – Regional Lymph Nodes

Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph nodes metastasis
N1	Regional lymph node metastasis

Regional lymph nodes:
- Cervical esophagus:
  - Scalene
  - Internal jugular
  - Upper and lower cervical
  - Periesophageal
  - Supraclavicular

- Intrathoracic esophagus:
  - Upper periesophageal
  - Subcarinal
  - Lower periesophageal
  - Mediastinal lymph nodes
  - Perigastric lymph nodes

M – Distant Metastasis

Mx Distant metastasis cannot be assessed
M0 No distant metastasis
M1 Distant metastasis

Tumors of the upper thoracic esophagus:
M1a Metastasis in cervical lymph nodes
M1b Other distant metastasis

Tumors of the mid-thoracic esophagus:
M1a Not applicable
M1b Non-regional lymph nodes or other distant metastasis

Tumors of the lower thoracic esophagus:
M1a Metastasis in celiac lymph nodes
M1b Other distant metastasis

pTNM – Pathological Classification

The pT, pN and pM categories correspond to the TNM categories (UICC 2005).

AJCC/UICC Stage Grouping for Esophageal Cancer (AJCC 2002, UICC 2005)

Cancer		
Stage 0	-	Tis N0 M0
Stage I	-	T1 N0 M0
Stage IIA	-	T2 N0 M0
		T3 N0 M0
Stage IIB	-	T1 N1 M0
		T2 N1 M0
Stage III	-	T3 N1 M0
		T4 Any N M0
Stage IV	-	Any T Any N M1
Stage IVA	-	Any T Any N M1a
Stage IVB	-	Any T Any N M1b

2. LIST OF PAPERS

Paper I
Goscinski MA, Suo ZH, Nesland JM, Florenes VA, Giercksky KE.
Dipeptidyl peptidase IV expression in cancer and stromal cells of human esophageal squamous cell carcinomas, adenocarcinomas and squamous cell carcinoma cell lines. APMIS 2008;116:823-31.

Paper II
Goscinski MA, Suo ZH, Nesland JM, Chen WT, Zakrzewska M, Wang J, Zhang S, Florenes VA, Giercksky KE.
Seprase, DPPIV and uPA expression in dysplasia and invasive squamous cell carcinoma of the esophagus. A study of 229 cases from Anyang Tumor Hospital, Henan Province, China. Oncology 2008;75:49-59.

Paper III
Goscinski MA, Suo ZH, Florenes VA, Vlatkovic L, Nesland JM, Giercksky KE.
Fap-α and uPA show different expression patterns in premalignant and malignant esophageal lesions. Ultrastruct Pathol 2008;32:89-96.

Paper IV
Goscinski MA, Larsen SG, Warloe T, Stoldt S, Nesland JM, Suo ZH, Giercksky KE.
Adenocarcinomas on the rise – does it influence survival from oesophageal cancer? Scand J Surg 2008, submitted.

3. GENERAL INTRODUCTION

Once the diagnosis of cancer is established, it is of utmost importance to determine whether the disease is local or already has spread to lymph nodes or distant organs (Fidler & Ellis 1994). Some scientists suggest that in nearly 50% of patients, surgical excision of primary malignant tumors is not curative because metastases have already occurred by that time (Sugarbaker et al. 1977, Sugarbaker 1979, Fidler & Balch 1987). Currently, metastases are the cause of 90% of human cancer mortality (Mehlen et al. 2006). Recent studies and clinical experiences show that metastases without any doubt are the most dreaded aspect of cancer.

In the 20th century, many different hypotheses about metastases were put forward. One of the first approaches was Stephen Paget's "seed and soil" hypothesis (Paget 1889), describing organs that are affected by disseminated cancer. Later, scientists such as James Ewing, Dale Rex Coman, Irving Zeidman, Barbara Lucke and others, suggested different theories about the origin of metastases (Fidler 2003). Isaiah J. Fidler reported in 1970-1973 that metastasis can result from the survival of only a few tumor cells (Fidler 1970, Weiss 1986, Fidler 1990). After the year 2000, research concerning tumor progression and metastasis development confirmed the theory that at the time of diagnosis, many human tumors are heterogeneous and include numerous cell subpopulations. The tumors also contain the so-called "cancer stem cells", which display different biological characteristics and metastatic potential (Bonnet & Dick 1997, Fidler 2003).

Today, research on malignant expansion is in progress and mainly concentrates on early cancer diagnosis and cancer therapy directed against host factors – angiogenesis and organ growth factors, as it is believed to provide a basis for treatment that will give better results than conventional therapy (Simone et al. 1998, Fidler 2003).

As mentioned above, there are many hypotheses and approaches trying to shed light upon the process of neoplastic disease development. Some of the theories focus particularly on the formation of metastases from the primary tumor through an exceedingly complex process. It includes a series of sequential steps such as invasion of adjacent tissue, intravasation, transport through the circulatory system and arrest at a secondary site, extravasation and growth in a secondary organ (Folkman 1986, Liotta 1986, Nicolson 1988). To complete this process, degradation of the extracellular matrix (ECM) is decisive (Folkman 1986, Takino 2007). Basic and clinical research has therefore been concentrated on the role of tumor-associated proteolytic systems – proteases and anti-proteases, particularly membrane proteases. Some of the most extensively studied proteases are dipeptidyl peptidase IV (DPPIV), urokinase-type plasminogen activator (uPA) and, recently, seprase (surface expressed protease, also known as fibroblast activation protease α (FAP-α)) (Vivier et al. 1991, Morimoto et al. 1994, Hansen et al. 1994, Rettig et al. 1994, Scalan et al. 1994, Mathew et al. 1995, Chen 2003, Chen & Kelly 2003).

Many studies have shown that in several tumors the expression of membrane proteases in cancer cells or stromal cells adjacent to cancer sites correlates with an

increasing tumorigenicity (Nishino et al. 1988, Scalan et al 1994, Nekarda et al. 1998, Okada et al. 2003, Iwasa et al. 2003, Kikkawa et al. 2005). However, the pathophysiologic significance of the serine proteases in esophageal carcinoma has not yet been fully elucidated.

Epidemiology of esophageal cancer

Esophageal cancer is turning into one of the more common cancers in the world. The incidence shows great geographical variations, but on the whole it is stated to be number six in frequency (Stewart & Kleihues 2003, Siewert et al. 2004, NCI 2006). The majority of cases are diagnosed in developing countries, where it is the fourth most frequently occurring cancer. The area with the highest reported incidence is known as the "Esophageal cancer belt of South-Central Asia", which extends from Turkey through northern Iran, Afghanistan and southern Russia to northern China (Ghadirian 1982, Saidi 1999, Hajian 2002). The invasive cancer incidence rate in these areas is more than 200 per 100 000 inhabitants, compared to Europe and the USA where it is 2.6-11.1 cases and 2.6-5.9 cases per 100 000 inhabitants respectively, with considerable racial and regional differences (NCI 2006). In particular, the area located in the southern part of the Taihang Mountains on the borders of Henan, Shansi and Hopei provinces in China has one of the highest incidence and mortality rate for esophageal carcinoma in the world. In Linxian county in Henan province, the age-adjusted mortality rate for esophageal carcinoma has been reported to be 151/100 000 for males and 115/100 000 for females (Lu et al. 1988, Stoner et al. 2001, He et al. 2008).

There are two different histological types of esophageal carcinoma: squamous cell carcinoma (SCC) and adenocarcinoma (AC). In Western countries, the number of SCCs and ACs is almost equivalent, while in developing countries, SCC is the most prevalent one (Pera et al. 1993, Devesa et al. 1998, Blot & McLaughlin 1999, Daly et al. 2000, Corley et al. 2001, Parkin et al. 2002, Pera et al. 2005, de Jonge et al. 2006, Yee et al. 2007, Holmes et al. 2007).

Since the entire esophageal tract is normally lined with squamous epithelium, SCC can occur anyplace along the length of the esophagus. AC, on the other hand, starts in tissue lined by columnar epithelium, which normally does not cover the esophagus. In order for an AC to develop, columnar epithelium must replace an area of squamous epithelium in a metaplastic process (Barrett's metaplasia), often followed by dysplasia (non-invasive neoplastic transformation with a potential for malignant progression). This occurs mainly in the lower esophagus, where most ACs are found (Stein et al. 1993, Cote et al. 2003, de Jonge et al. 2006).

Squamous cell carcinoma

The esophagus is normally lined with stratified squamous nonkeratinizing epithelium (Fig.1A) (Norton et al. 2000). SCC arises from this epithelial layer, probably in response to chronic toxic irritation. In many cases, esophageal SCC is the end result of a progression through increasingly severe degrees of dysplasia to carcinoma *in situ*, resulting in invasive carcinoma (Fig. 1B). Clinical and epidemiological studies have confirmed that squamous epithelial dysplasia of the esophagus is a precancerous lesion and that approximately 70% of patients with squamous dysplasia later develop SCC (Mukada et al. 1978, Munoz et al. 1982, Mandard et al. 1984, Kuwano et al. 1988).

Epithelial dysplasia has previously been classified into low-, moderate- and high grades (Oehlert et al. 1979, Riddell et al. 1983, Tosi et al. 1989, de Dombal et al. 1990, WHO 1990, Lewin & Appelman 1996). Low grade dysplasia usually affects less than half of the epithelial thickness, while high grade dysplasia affects more than half of it. Moderate grade dysplasia is borderline or intermediate between the two other grades. Sometimes, separating one grade from another is difficult, as the lines of demarcation are not always clear. Nowadays, many chose to use thus the two-grade system, low- and high grade dysplasia (Riddell et al. 1983, Tosi et al. 1987 & 1989, Rubio et al. 1989, de Dombal et al. 1990, Burke et al. 1991).

When it comes to the transition from high grade dysplasia to carcinoma *in situ*, no clear distinction can be made either macro- or microscopically. Both stages display a similar histological pattern and probably require similar surveillance and treatment (Lewin et al. 1998).

Microscopic features

Preinvasive lesions are characterized by cellular atypia and abnormal intraepithelial architecture. These features progress until atypical cells have spread to all epithelial layers and the architecture no longer can be identified. These characteristics are clearly visible in high grade dysplasia (carcinoma *in situ*), whereas low grade dysplasia still has a component of differentiated squamous cells in the upper layers, and abnormal cells are limited to the lower half of the epithelium (Crissman et al. 1987 & 1989, Mills et al. 2000, WHO 2000).

The histologic appearance of invasive SCC is related to invasion of atypical cells into lamina propria or beyond it (Schlemper et al. 1997). Usually, a large pushing front of numerous cohesive squamous cells can be observed, but the cells may also invade as small cell nests or as individual cells infiltrating through the stroma. Invasive carcinomas generate a surrounding inflammation, where a mixture of various leukocytes can be observed (Fig. 1B and 1C) (Crissman et al. 1987 & 1989, WHO 2000).

Risk factors

Use of tobacco and alcohol are among the highest risk factors regarding SCC. Consumption of salty and spicy food, as well as low fiber intake have also been positively identified as risk factors for developing SCC (Soler et al. 2001, Enzinger & Mayer 2003, Syrjänen 2002, Green et al. 2003). Further risk factors are summarized in Table 1.

Adenocarcinoma

In contrast to SCC, the incidence of AC nowadays is rising rapidly to the point where this tumor accounts for 50% or more of all esophageal cancers in the USA and other industrialized countries (Lagergren et al. 1999, Bollscheweiler 2001, de Jonge et al. 2006, Merry et al. 2007, Hashibe et al. 2007). ACs occur almost exclusively in the distal part of the esophagus. The tumor is first seen as a thickened plaque-like white mucosa. Larger lesions form white exophytic polypoid masses with well demarcated borders. Occasionally, carcinomas appear papillary or may be multifocal. AC spreads through the esophagus into periesophageal tissues (Fenoglio-Preiser et al. 1989).

Microscopic features

The majority of ACs is classified as low-, moderate to well differentiated carcinomas and form glands with columnar epithelium. The epithelial cells contain nuclei with a coarse chromatin pattern, nucleoli and cytoplasm in which mucin can be found (Fig. 1F). A minority of tumors displays the diffuse type pattern with signet ring cells (Petterson 1932, Adams et al. 1945, Spin 1973, Thompson et al. 1983, Thurberg et al. 1999, Nakagawa et al. 2000).

Risk factors

The major acknowledged risk factor for AC is the pathological and clinical alteration of the lower esophagus, also known as Barrett's esophagus, coexisting with gastroesophageal reflux disease (GERD), wherein columnar epithelium replaces the squamous epithelium that normally lines the distal esophagus (Lagergren et al. 1999, Heath et al. 2000, Ye et al. 2004, Nakajima & Hattori 2004, Oberg et al. 2005, Layke, et al. 2006). Other risk factors are summarized in Table 1.

Table 1. Risk factors for esophageal carcinoma.

SCC	AC
Tobacco smoking*	GERD
Alcohol consumption	Barrett's esophagus
Consumption of salt-cured, salt-pickled and moldy food**	High BMI
Low fiber intake	Tobacco smoking*
Consumption of hot beverages	Use of NSAID
Achalasia	Work in stooped posture
Previous head and neck cancer	Genetic alterations
Plummer-Vinson syndrome	
HPV (human papilloma virus) infection	
Genetic alterations	

* Nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, aldehydes and phenols

** Nitrosamines and fungal toxins

Barrett's esophagus

The importance of Barrett's esophagus rests on the fact that local occurrence of columnar metaplasia can develop into AC. The concept of Barrett's esophagus has changed significantly since its first description by Numan R. Barrett in 1950 and 1957 (Barrett 1950 & 1957). In 1950, in "Chronic Peptic Ulcer of the Oesophagus and Oesophagitis", Barrett reviewed several published cases, looked at preserved pathologic specimens and found that esophageal ulcers were surrounded by columnar mucosa. He concluded that "these cases were examples of congenital short esophagus – in which part of the stomach extends upwards into the mediastinum, or even to the neck, and that in this stomach a typical chronic gastric ulcer can form". He suggested that the ulcer was a separate entity from reflux esophagitis (Barrett 1950, Cameron 2001). In 1957, Barrett revised his theory and noted that the columnar lining was a continuous sheet extending from the esophago-gastric junction upwards, and that it was thus columnar cell-lined esophagus extending into the mediastinum and not the stomach (Barrett 1957). As a result of these observations, the term "Barrett's esophagus" has become well established in medical literature to indicate columnar metaplasia of the distal esophagus associated with chronic GERD. Ever since, several different concepts of Barrett's esophagus have emerged (Cohen et al. 1963, Trier 1970, Bremner et al. 1970, Naef & Savary 1972, Iascone et al. 1983, Gillen et al. 1988).

Based on the potential development of malignancy, the heterogenous spectrum of Barrett's metaplastic mucosa is classified into two distinct types: columnar epithelium, with specialized intestinal metaplasia (SIM), including goblet and columnar non-goblet cells, and columnar epithelium, without specialized intestinal metaplasia and lacking goblet cell-type elements (Barrett 1957, Siewert & Dittler 1993, Koppert et al. 2005, Fléjou 2005). The goblet cells are presently regarded as the hallmark in the histological identification of Barrett's esophagus and in the selections of high-risk patients for endoscopic surveillance (Chaves et al. 1999). The columnar epithelium with SIM is the most common and distinctive epithelium type found in Barrett's esophagus (Fig. 1D) (Spechler et al. 1994, Spechler 2003).

Dysplasia in Barrett's esophagus

Dysplasia in Barrett's esophagus can be classified as low-, moderate or high grade. In low grade dysplasia, simple tubules with little branching can be demonstrated. Atypical cells are present in the glands, and the epithelium is usually pseudostratified. Mitotic figures usually are sparse but can be present in the superficial half of the mucosa (Lewin 1998, Goldblum 2003).

High grade dysplasia (carcinoma *in situ*) shows severe cellular atypia and varying degrees of abnormal architecture. The nuclei are often enlarged, vesicular with irregularly clumped chromatin and contain large, distinct and irregular nucleoli (Fig. 1D and 1E) (Smith et al. 1984, Schmidt et al. 1985, Spechler & Goyal 1986, Reid et al. 1988). Moderate grade dysplasia is a borderline condition between low- and high grade dysplasia.

Macroscopic appearance of Barrett's esophagus

Endoscopy reveals columnar epithelium in the distal esophagus with a characteristic red color which contrasts sharply with the pale appearance of adjacent squamous epithelium (Spechler & Goyal 1996). Although endoscopic examination can usually distinguish columnar epithelium from squamous epithelium, it is difficult to tell apart different types of columnar epithelium by endoscopic appearance alone. SIM and gastric columnar epithelium can only be distinguished by histological examination of biopsy specimens. Gastric epithelium may normally line a short segment of the distal esophagus. Thus, endoscopists usually diagnose Barrett's esophagus only when columnar epithelium extending well above the gastroesophageal junction can be verified. The criteria for diagnosing Barrett's esophagus based on the length of the columnar lining have varied, ranging from 2-5 cm or more, measured above the gastroesophageal junction. Nowadays, we distinguish only two types of Barrett's esophagus: the long segment, where columnar lining extends more than three cm from the gastroesophageal junction, and the short segment, where columnar lining extends up to three cm from the gastroesophageal junction (Schnell et al. 1992, Levine et al. 1993, Spechler 1994 & 2003, Hirota et al. 1999, Spechler & Goyal 1996).

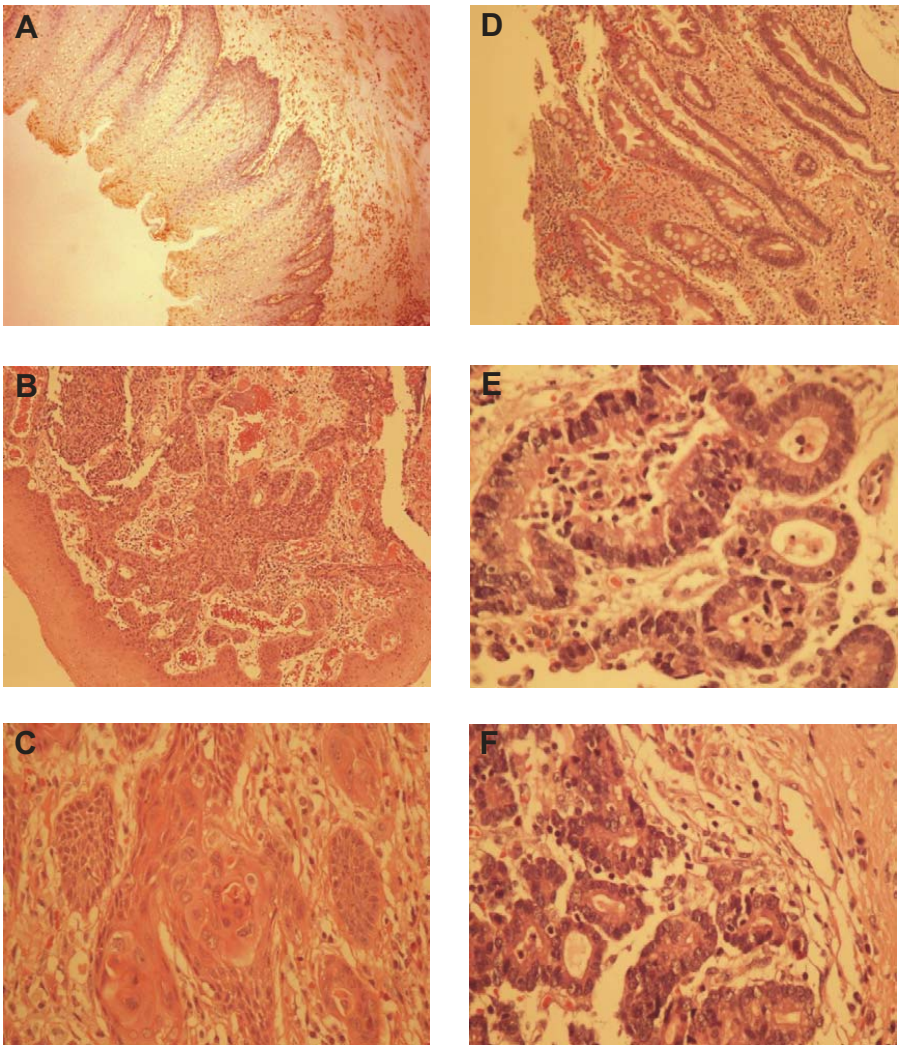

Figure 1. Microscopic illustrations of SCC and AC (original magnification x 200).
Normal esophageal epithelium (A). Transition of normal esophageal epithelium toward SCC (B). Well differentiated SCC (C). Barrett's esophagus with intestinal metaplasia within columnar epithelium (D). High grade dysplasia within columnar epithelium (E). Moderately differentiated AC (F).

Early esophageal cancer

Early-stage esophageal cancer is almost asymptomatic with minimal findings in endoscopy. However, a risk for malignant spreading into vessels and lymph nodes is present even in small tumors (Peters et al. 1994, van Sandick et al. 1998, Stein et al. 2000 & 2005, Buttar et al. 2001).

The accepted definition of early esophageal cancer is based on two related elements: superficial extension of the tumor in mucosa and submucosa, and complete or almost complete absence of lymph node metastasis. Most cases correspond therefore to stage I cancer (T1N0M0), according to the TNM/AJCC/UICC staging system (see above) (AJCC 2002, UICC 2005).

This definition does not apply to all superficial SCC tumors (T1). The lymph node involvement is negligible only when the tumor is limited to the mucosa. There are three successive phases in the invasion of mucosa: intraepithelial cancer (carcinoma *in situ*), without affection of the basal membrane; microinvasive cancer with invasion into lamina propria; and intramucosal / transmucosal cancer, invading the muscularis mucosa. No spreading to lymph nodes is associated with intraepithelial cancer, and only a small number of lymph nodes can present metastasis in microinvasive cancer. Intramucosal cancer presents a higher degree of lymph node metastases and reaches the submucosal mucous glands through intraductal propagation (Hölscher et al.1997, Ell et al. 2000, Rice et al. 2001, May et al. 2002, Stein et al. 2003).

Severe dysplasia is considered as carcinoma *in situ* and is often found in patients with multicentric esophageal cancer. Tumors extending into the submucosa have a risk rate for lymphatic invasion varying from 30% to 50%. Submucosal SCC cannot be considered as an early cancer, but must instead be treated as an advanced cancer (Streitz et al. 1991, Nigro et al. 1999, Tajima et al. 2000, Buskens et al. 2004). In AC that has developed in columnar cell-lined esophagus, a clear-cut distinction between superficial (T1) and non-superficial (T2) cancer is based on whether it invades the muscularis propria or not (Lambert R, 1995, Hölscher et al.1997).

Esophageal cancer diagnostics

Direct diagnostic techniques:
- Esophagoscopy with biopsy followed by a pathological evaluation
- Barium X-ray
- Computed tomography with multi-slice technique

Diagnostic techniques used to determine the extent of disease:
- Endoscopic ultrasound
- PET and PET/CT scans
- Bronchoscopy
- Thoracoscopy and laparoscopy

Esophageal cancer research areas

It is well known that several environmental factors can lead to the development of esophageal carcinoma. Environmental carcinogens are able to affect the genetic material of host cells, generating an abnormal regulation of multiple genes (Stoner et al. 2007). Genetic alterations observed in esophageal carcinomas are based on alterations in tumor suppressor genes leading to uncontrolled cell proliferation and terminating apoptosis, loss of cell cycle control as a result of disruption of G1/S cell cycle checkpoints and changes in oncogene functions leading to deregulation of cell signaling pathways (Stoner et al. 2007). Several previous studies have reported numerous genetic alterations associated with esophageal carcinomas, as summarized in Table 2 (Lu et al. 1988, Hollstein et al. 1988 & 1991, Jiang et al. 1993, Guo et al. 1993, Gao et al. 1994, El-Rifai et al. 1998, Moskaluk 1998, Tanaka et al. 1999, Hiyama et al. 1999, Xing et al. 1999, van Dekken 1999, Raida et al. 1999, Zimmermann et al. 1999, Kimura et al. 1999, Lu 2000, Mandard et al. 2000, Selaru 2002, Kuwano et al. 2005).

The discovery of new tumor cell markers, e.g. growth factor receptors, angiogenetic and apoptotic factors or transmembrane proteases, has become an important current research topic (Table 3) (Gottlinger et al. 1986, Robaszkiewicz et al.1991, Reid et al. 1992 & 2000, Traweek et al. 1993, Galipeau et al. 1996, Teodori et al. 1998, Hanahan & Weinberg 2000, Lam 2000, Mandard et al. 2000, Blant et al. 2001, Xu Y et al. 2002, Klein et al. 2002, Yu et al. 2003, Hosch et al. 2003). The expression differences in molecular markers in premalignant and malignant tumor stages, as well as in various histological tumor types are essential for the development of more effective and early diagnostic methods and less harmful therapies for esophageal carcinoma.

In combination with surgery, gene therapy, immunotherapy and new types of chemotherapy, including tyrosine kinase inhibitors or examination of new combinations of existing drugs with irradiation (multimodal therapy), are the current treatment trends (Shaheen & Ransohoff 2002, AJCC 2002, Enzinger & Mayer 2003, Swisher et al. 2003, Burmeister et al. 2005, Kleinberg et al. 2004, Koshy et al. 2004, Posner et al. 2005, Souza & Spechler 2005, ACS 2008).

Table 2. Genetic alterations associated with esophageal carcinoma (modified after Stoner & Gupta 2001).

SCC
p53 mutations
Loss of p16MST1 and / or p15, and / or RARβ and disabled-2 (DAB-2)
Amplification of INT-2, EGFR, cyclin D_1 and c-Myc
Altered expression of the cyclin D1
Altered expression of apoptosis related genes: bcl-2, caspase 3, TRAIL, Fas-L, Fas
Elevations in hTERT, BMP-6, iNOS, COX-2 and β-catenin levels
Loss of heterozygosity on chromosomes 1p, 3p, 4, 5q, 9, 11q, 13q, 17q and 18
Enhanced expression of the transcription activator, NFκB
AC
Gains of chromosomes 6p, 7pq, 8q, and 17q
High-level amplifications at 8q23~q24.1, 15q25, 17q12q21 and 19q13.1
Losses of chromosomes 4pq, 5q, 18q, 19p, 20, 21, and Y

Table 3. Molecular markers in esophageal cancer (modified after Stoner & Gupta 2001).

Markers
Growth factor receptors
EGFR
HER2/neu
Angiogenetic factors
Cox-2
TP
VEGF
b-FGF
TGF-α, TGF-β
Cell cycle regulators
Cyclin D1
p21, p27, p53
Apoptotic factors
Bcl-2
Bcl-X
P16
Survivin
DNA repair system
ERCC3
Matrix metalloproteinases
MMP-2, MMP-7, MMP-9

Treatment of esophageal carcinoma

Single modality treatments

Surgery

Surgical resection has long been the mainstay of curative treatment and remains the standard treatment for stage T1-T3, N0, M0 tumors. It is contra-indicated in locally advanced tumors (T4) and when lymph node and distant metastases have developed (Fumagalli 1996, Veuillez et al. 2007). Total or partial esophagectomy is most commonly performed on esophageal and gastric cardia carcinomas, as well as in patients with Barrett's esophagus associated with severe dysplasia, undilatable strictures and benign, obstructing tumors (Fumagalli 1996).
Usually, resection of the thoracic esophagus is made by a right- or left-sided transthoracic 'en bloc' resection. A transhiatal resection is an option for cancer of the distal part of the esophagus (Skinner 1983, Orringer 1984, Killinger et al. 1996, Veuillez et al. 2007).

Esophageal cancer involves a risk for lymph node metastasis development, and the quality of the lymphadenectomy associated with esophagectomy is therefore important. Two-field lymphadenectomy (thoracic and abdominal) for cancers from the distal and middle part of the esophagus and three-field lymphadenectomy (thoracic, abdominal and cervical) for cancers of the proximal part of the esophagus are recommended (Fumagalli 1996, Lerut et al. 2004, Veuillez et al. 2007).

The results depend on the extension of the disease, possible co-morbidities and the surgeon's experience. The post-operative mortality rate currently ranges from 4% to 10%, depending on the surgical centers (patient volume and team experience) (Collard et al. 2001, Bumm & Wong 1994). After R0 resection, the reported 5-year overall survival rate is greater than 95% for stage 0, between 50% and 80% for stage I, 30% and 40% for stage IIA, 10% and 30% for stage IIB and between 10% and 15% for stage III (Enzinger & Mayer 2003, NCI 2006, Veuillez et al. 2007).

Endoscopic Mucosal Resection

This method is restricted to superficial cancers limited to the mucosa, submucosa or precancerous lesions and is not recommended for tumors invading the muscularis mucosa or if lymph node metastasis is suspected. The procedure is usually performed after confirmation of the extent of the lesion by iodine staining. An endoscopic mucosal resection (EMR) tube with a snare which seizes the lifted mucosa including its lesion is used. Finally, the mucosa is resected by electric current (Makuuchi et al. 1992, Yokoyama et al. 1995, Makuuchi1996).

The rate of success is more than 90% when indications are respected, and the immediate morbidity rate is low (perforation less than 1%, death 0.1%). However, delayed complications are more common and are reported in about 6% of cases

(stenosis, recurrences, bleeding) (Kodama et al. 1998, Inoue 1998, Veuillez et al. 2007).

The problems arise when the lesion is not completely removed and additional excision is needed such as piecemeal resection carrying the possibility of tumor tissue implantation. In these cases, supplementary treatments such as photodynamic therapy and radiofrequency ablation (RFA) are often warranted.

Laser therapy

Laser therapy is today considered a palliative endoscopic therapy (Spencer et al. 2002). The tumor is vaporized or coagulated under direct vision with no mechanical stress on the esophageal wall. This technique is suitable for patients with exophytic tumors. Successful tumor recanalization can be achieved in more than 90% of the patients, and a subsequent return to eating solids can be obtained in the majority. Laser therapy does, however, need to be repeated every 4–6 weeks as the tumor re-grows. This problem may be solved by combining it with adjuvant radiotherapy (Spencer et al. 2002). Palliative laser therapy has largely been replaced by self-expanding stents or been combined with these.

Photodynamic therapy

Photodynamic therapy (PDT) is generally used with the intent of curing early stages of esophageal cancer and as a palliative treatment for advanced and recurrent cancers. PDT is initiated with the administration of a photosensitizer (sodic porfimere, 5-ALA, meta-tetra hydroxyphenyl chlorine) and local tumor treatment is activated later using appropriate laser monochromatic light (Sibille et al. 1995, Veuillez et al. 2007).

The advantage of some of the PDT drugs is that they destroy cancer cells with less harm to normal cells. One drawback is that the photosensitizer must be activated by laser light; therefore only superficial cancers can be treated. The light cannot reach cancers that have expanded deeper into the esophageal wall or spread to other organs. As a palliative therapy, PDT does not eliminate all cancer tissue, but partially removes it and relieves the dysphagia.

When PDT is used on superficial cancers, the rate of success for this technique is approximately 100% but, depending of the drug used, includes a risk for symptomatic stenosis in nearly one-third of the cases (Barr 2003). In a single study, the 5-year survival rate was 74% (Sibille et al. 1995).

PDT, EMR and RFA, either alone or in combination, are simple, efficient and non-mutilating procedures that could be used on all localized lesions defined by a battery of markers to be precancerous even in patients with severe co-morbidity.

Multimodal treatment

Radio- and chemotherapy

When surgery is contra-indicated and the patients have no signs of distant metastases, infiltrating esophagus cancers may be treated using radio- and chemotherapy (Veuillez et al. 2007). The first report, by Herskovic in 1992, demonstrated that combined platinum-based radio- and chemotherapy treatment (50 Gy + five days with infusion of 5-fluorouracil (5-FU) and one day of cisplatin), followed by two cycles of the same chemotherapy regimen, generated better results than external irradiation alone. The radio- and chemotherapy combination resulted in a 25% 5-year overall survival rate compared to 0% for radiotherapy alone (Herskovic et al. 1992, Al-Sarraf et al. 1997). This study established the radio- and chemotherapy combination as a standard treatment for localized esophageal cancer (Stages II and III) (Veuillez et al. 2007). Other investigations, however, showed that there was no benefit in terms of overall survival in favor of secondary resection for patients responding to radio- and chemotherapy combination treatment (Stahl et al. 2005, Bedenne et al. 2007).

Recently, recommendations on the multimodal treatment (surgery and chemoradiation) of esophageal carcinoma have been published by Seitz and have been proposed as a general strategy for treatment of esophageal cancer (Seitz et al. 2006).
Superficial cancers (*in situ* or T1-m1 or T1-m2, where "m" stands for the degree of infiltration into the mucosa) do not require multimodal treatment. Tumors covering more than two cm may be treated by mucosectomy. However, if the pathological examination reveals a more invasive tumor (T1-m3 or T2) with a risk for lymph node metastasis development, additional treatment is necessary and esophagectomy or radio- and chemotherapy combination has to be discussed depending on the condition of the patient.

Stage I (T1–T2, N0) invasive intrathoracic cancers are subject to surgical resection as standard treatment. In case of lymph node metastasis, adjuvant chemotherapy may be considered. In case of contra-indications to surgical resection, a radio- and chemotherapy combination may be appropriate.

Stage II (T1N1, T2N1, T3N0) invasive intrathoracic cancers usually undergo surgical resection preceded by neoadjuvant chemotherapy as standard treatment. Contra-indications to surgery entail the administration of radio- and chemotherapy.

Stage III (T3N1, T4N0-N1) intrathoracic invasive SCCs are subject to radio- and chemotherapy as standard treatment. In case of incomplete response or early recurrence, a salvage surgical excision must be considered.

Regarding AC, neoadjuvant chemotherapy before and after surgical resection is a valid option and by many considered the standard treatment (Cunningham et al. 2006). Locally inoperable cancers may be treated with a radio- and chemotherapy combination.

Radio- and chemotherapy combination is recommended when surgical resection is not possible for cervical cancers.

Non-operable patients without metastasis usually undergo a radio- and chemotherapy combination or receive an esophageal stent and occasionally an endotracheal stent in addition. The choice of treatment depends on the presence of esophago-tracheal or bronchial fistulae.

Patients with metastatic disease may be treated with radiotherapy or radio- and chemotherapy. An esophageal stent followed by chemotherapy is another option.

Common to all regimens is a disappointing low 5-year survival rate when clinical signs of cancer initiate treatment. Future strategy directed against symptoms (reflux) and dysplasia signs in premalignant lesions, combined with markers of malignant development and local treatment could improve the survival rate.

Palliative treatment

Stenting has become the treatment of choice for strictures with inoperable esophageal cancer (Guo et al. 2007 & 2008). However, stricture recurrence remains a challenge after a stent placement. Previously, endoscopic insertion of rubber stents was the first choice, but more recently, expanding metal stents have been introduced, as they are easier and safer to insert and less initial dilatation is required. Once in position, they expand across the tumor, but as experience shows, the swallowing quality is often not better, and the patients may have as many problems as with the rubber stents (Spencer et al. 2002).

Tumor invasion and development of metastases

Tumor invasion into the collagenous matrix and metastases represent the main problems in the treatment of carcinoma patients. The majority of patients with newly diagnosed carcinomas already present clinically detectable metastases (Fidler et al. 1978, Fidler & Hart 1982, Fidler & Balch 1987, Weiss 2000). In the tumor invasion process, cancer cells infiltrate the adjacent ECM by using several types of matrix degrading enzymes such as metalloproteinases, cysteine-, aspartic-, threonine and serine proteinases (Fig. 2) (Duffy 1987, Zukker 1988, Brunner & Preissner 1994, Keppler et al. 1994, Sloane et al. 1994, MacDougall & Matrisian 1995, Birkedal-Hansen 1995, Hewitt & Danø 1996). These enzymes are produced by the tumor cells and / or the surrounding host cells, and they cooperate with other proteins, for example integrins, cadherins and immunoglobulins, which may facilitate the dissolution process of the ECM (Hynes 1992, Natali et al. 1992, Morino et al. 1995, Takeichi 1991 & 1995 Rucklidge et al. 1994).

Next, neovascularization takes place, and cancer cells subsequently invade blood vessels in order to move to another organ. Adherent to the blood vessel walls,

malignant cells leave the primary circulation and migrate into the neighboring tissue, in a process called extravasation. They thus establish a new tumor site in the organism. The metastatic process is characterized by a highly selective competition, favoring the survival of a small subpopulation of metastatic cells (Liotta 1986, Fidler & Hart 1982). The metastatic subpopulation is abundant in the primary tumor tissue early in its growth, but less than 0.01% of malignant cells entering circulation actually form metastases (Kerbel et al. 1990, Fidler & Ellis 1994).

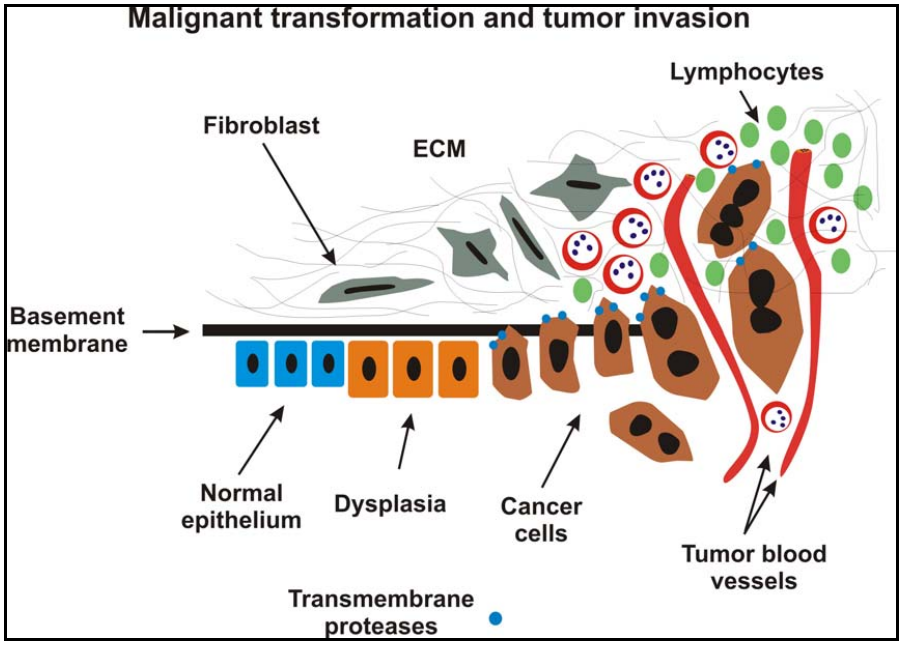

Figure 2. Invasive carcinoma development (modified after Liotta & Kohn 2001).

Membrane proteases

Membrane-bound proteases are widely spread among the different cell systems. Their expression in particular cell types is finely regulated, reflecting the specific functional cell implications and engagement in defined physiological pathways (Sedo et al. 2001). It has been reported that the proteases play a crucial role, both as effectors and regulatory molecules in protein turnover, ontogeny, inflammation, tissue remodeling, cell migration and tumor invasion (Sedo et al. 2001).

Several families of membrane proteases have been identified on the basis of their proteolytic activities, biologic functions and structural organization (Chen 2003, Chen & Kelly 2003):

1. Membrane-type matrix metalloproteinases (MT-MMPs)
2. Disintegrin and metalloproteases (ADAM family)
3. Meprins
4. Secretases
5. Metallo- and serine peptidases (SIMP)

It is generally known that a given membrane protease may have several functions (diversity) and that more than one protease or protease family may mediate the same function (redundancy) (Bauvois B 2001). Soluble counterparts of some membrane proteases have been found intracellularly as well as in extracellular fluids, including blood plasma (Rettig et al. 1988, Sedo et al. 1996, Chiravuri et al. 1999, Abbott et al. 2000, Tang et al. 2000, Goldstein & Chen 2000, Chen 2003).

Localization of enzymes is critical for their function in cellular activities. It has been shown before that MT-MMPS and SIMP may have a prominent role in processing soluble factors as well as in degrading the components of the ECM (Sato et al. 1994). This study aims to examine this area in more detail and is focused on three members of serine protease family which are introduced below.

Seprase / FAP-α

FAP-α was first identified as an inducible antigen expressed on reactive stromal fibroblasts (Rettig et al. 1988 & 1993, Garin-Chesa et al. 1990). In parallel, seprase was originally isolated as a 170-kDa transmembrane protease from the malignant melanoma cell line LOX (Aoyama & Chen 1990, Monsky et al. 1994, Kelly et al. 1994). Further molecular cloning of FAP-α and seprase revealed the identical gene and protein (Scanlan et al. 1994, Piñeiro-Sánchez et al. 1997, Chen & Kelly 2003, Chen 2003). The gene is localized on the long arm of chromosome 2 (2q23). Seprase / FAP-α is a type II transmembrane protein of 760 amino acids, anchored in the plasma membrane by a short transmembrane domain, intracellularly exposing an amino terminal sequence, whereas a catalytic domain with a carboxyl-terminus remains extracellularly (Park et al. 1999, Levy et al. 1999). It displays both prolyl dipeptidyl peptidase and gelatinase activities. The protease appears as a homodimer (170 kDa) containing two 97 kDa subunits. Glycosylation and dimerization of the enzyme are necessary for its protease activity (Sun et al. 2002, Kelly 2005).

In humans, seprase has been identified in tissue remodeling sites, reactive stromal fibroblast in 90% of malignant epithelial tumors and sarcomas, granulation tissue of healing wounds and fetal mesenchymal tissue. The immunopathological expression of seprase has previously been investigated in gastric and colon cancer, as well as in melanoma, ovarian and breast cancer and its overexpression was associated with malignant phenotype (Okada et al. 2003, Iwasa et al. 2003). Seprase is not expressed in normal adult human tissue (Garin-Chesa et al. 1990, Rettig et al. 1993, Scalan et al. 1994, Ariga et al. 2001).

Dipeptidyl peptidase IV (DPPIV, CD 26)

DPPIV was first identified in 1966 as glycylproline naphthylamidase (Hopsu-Havu & Glenner 1966). DPPIV is an integral membrane glycoprotein with type II topology. Native human DPPIV is a 110 kDa protein, while the active form is a 200-220 kDa homodimer that exhibits the dipeptidyl peptidase activity (Piazza et al. 1989, Johnson et al. 1993, Piñeiro-Sánchez et al. 1997). The DPPIV structure contains 766 amino acid residues. The human gene of DPPIV is localized on the long arm of chromosome 2 (2q24.3). Peptidase is expressed constitutively on brush border membranes of intestine and kidney epithelial cells and transiently in activated T-cells and migratory endothelial cells (Vivier et al. 1991, Yaron & Naider1993, Morimoto et al. 1994).

It was shown that DPPIV, in addition to its typical dipeptidyl aminopeptidase activity, may possess endopeptidase activity as well (Bermpol et al. 1998). DPPIV expression and activity was observed in numerous types of human malignancies (basal cell carcinoma, prostate-, ovarian- and thyroid carcinoma) as well as in blood plasma of cancer patients (Hirai et al. 1999, Wilson et al. 2005, Ozog et al. 2006). In general, higher DPPIV expression is associated with more aggressive tumor behavior.

In contrast, in ovarian carcinoma cell lines, DPPIV overexpression was associated with a decrease in invasive potential, change in morphology, reduction of intraperitoneal dissemination of carcinoma cells and prolongation of survival time in vivo (Kajiyama et al. 2002, Kikkawa et al. 2005). Functional studies have also demonstrated that loss of DPPIV expression during malignant transformation of melanocytes is accompanied by growth factor independence, whereas its experimentally induced re-expression leads to the suppression of tumorigenicity, reversal of a block in differentiation and re-emergence of requirements for exogenous growth factor (Wesley et al. 1999, Pethiyagoda et al. 2000).

Finally, a soluble form of DPPIV modulating the responsiveness of T-cells to specific antigens has been detected in blood plasma (Tanaka et al. 1994).

uPA

The serine protease family includes also uPA, a glycoprotein with a molecular weight of 55 kDa, known to operate extracellularly. It is activated on the cell surface, binding to a specific receptor (uPAR), which is linked to the plasma membrane and may form complexes with seprase, DPPIV and β1 integrin. uPA cleaves plasminogen to form active plasmin, breaking down most ECM components, including type IV collagen, laminin and fibronectin (Hansen et al.1994).

An elevated level of uPA has been involved in the development of invasiveness in numerous neoplasms, including breast-, ovarian-, gastric- and colorectal cancer, as well as SCC and AC (Nishino et al. 1988, Sier et al. 1991, Hewin et al. 1995, Torzewski et al. 1997, Nekarda et al.1998, Artym et al. 2002).

Serine protease complexes

Former studies have revealed that serine proteases are able to form complexes. Ghersi and Dong, for example, have shown that seprase and DPPIV are simultaneously involved in the degradation of the collagenous matrix (Ghersi et al. 2002). The two proteases form a complex localized at invadopodia of cells migrating on collagenous fibers that elicits both endo- and exopeptidase activities (Chen 2003). The complex, described as a 400 kDa molecule, develops into a potent ECM degrading factor (Piñeiro-Sánchez et al. 1997, Mueller et al. 1999, Ghersi et al. 2002).

Immunoprecipitation, immunofluorescence and cell surface crosslinking experiments demonstrated another seprase-DPPIV complex with $\alpha 3\beta 1$ integrins, additionally facilitating invasion into the collagenous matrix (Mueller et al. 1999, Chen 2003). Interestingly, $\alpha 3\beta 1$ integrin is even able to bind uPAR (Zhang et al. 2003). This binding demonstrates the possibility of creating a supramolecular complex consisting of seprase-DPPIV-uPAR/uPA- $\alpha 3\beta 1$ integrin, exposing common proteolytic activity (Scanlan et al. 1994, Ghersi et al. 2002, Artym et al. 2002, Kelly T 2005).

A recent study, performed on HUVEC, has shown a seprase-DPPIV complex (~ 820 kDa) localized at an invadopodia-like protrusion of endothelial cells involved in the invasion of the ECM (Ghersi et al. 2006). *In vivo* examination on invasive breast ductal carcinoma specimens has shown a distribution of the complex on the endothelial cells of capillaries, but not on large blood vessels, contributing thus to understanding the role of serine proteases in the angiogenetic process (Ghersi et al. 2006).

A simplified interaction model between the serine proteases, integrins and ECM is illustrated in Figure 3.

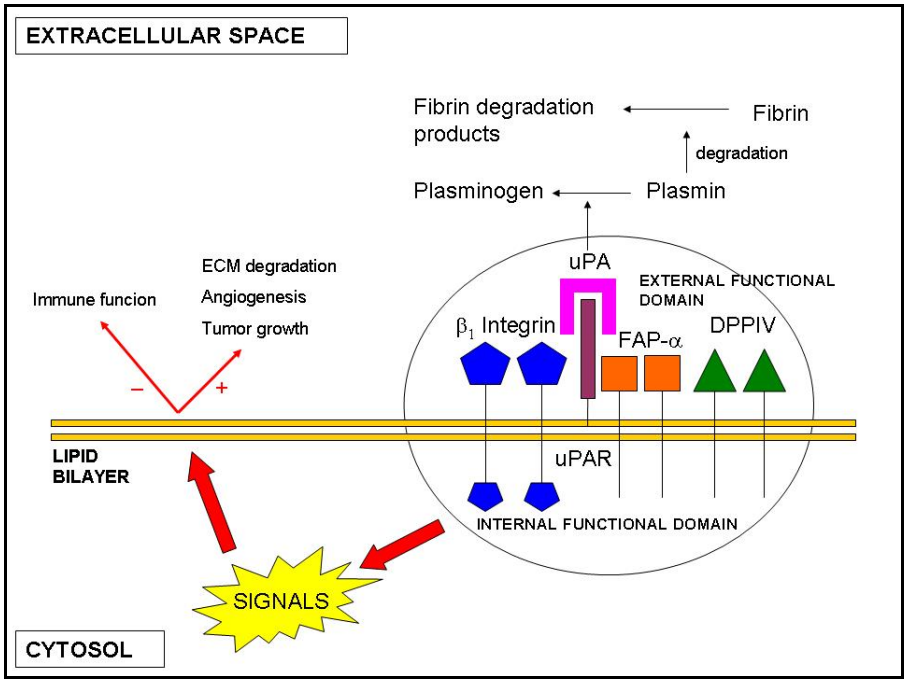

Figure 3. Possible serine protease complex. DPPIV, Fap-α, uPA / uPAR and β1 integrin with proteolytic, adhesive and signaling capabilities. The complex may act intra- and extracellularly, demonstrating a common enzymatic activity (modified after Kelly 2005).

4. AIMS OF THE STUDY

Little information about expression patterns of the serine protease family members in esophageal carcinomas was available when this study was initiated. Though uPA has previously been analyzed in esophageal carcinomas, the role of seprase and DPPIV in this carcinoma type is not yet known. No systemic study of expression patterns of all three serine proteases together in the upper gastrointestinal tract and their clinical association was available. Thanks to the fact that the study partly was a collaboration project between the Norwegian Radium Hospital, Rikshospitalet HF in Oslo, Norway and Anyang Tumor Hospital, Henan Province in China, we were able to collect extensive material from this particular tumor type. This gave us the unique opportunity to study the expression of serine proteases in normal, premalignant and malignant stages of both SCC and AC in order to better understand their involvement in neoplastic progression and the possible use as marker of malignant or metastatic potential.

Furthermore, from a clinical point of view, no complete report has been published until today, assessing patients treated at the Norwegian Radium Hospital, Rikshospitalet HF with esophageal carcinoma. Using our clinical databases and tissue material, we examined clinical and histopathological information taking into account all the esophageal cancer patients treated at the Norwegian Radium Hospital, Rikshospitalet HF between 1987-2007 in order to analyze epidemiology, occurrence of precancerous stages and treatment results.

The specific aims were to:

- Compare seprase, DPPIV and uPA expression in dysplastic and cancer cells of SCC, as well as in stromal cells adjacent to premalignant and malignant sites
- Examine seprase and uPA expression in Barrett's esophagus, dysplasia and AC, as well as in stromal cell bordering neoplastic alterations
- Compare DPPIV expression in cancer and stromal cells of SCC and AC
- Demonstrate stromal serine protease expression
- Describe and analyze clinicopathological features of the patients with esophageal carcinoma

5. MATERIALS AND METHODS

Patients and tissue samples

This study included two independent patient groups:

1. Norwegian patients who were diagnosed with esophageal dysplasia or esophageal cancer in the period from January 1987 until December 2007 and underwent periodic surveillance or received partial or complete treatment at the Surgical Oncology Department of the Norwegian Radium Hospital, Rikshospitalet HF in Oslo, Norway (Papers I, III and IV).
2. Chinese patients who were diagnosed with esophageal dysplasia or esophageal cancer between 2003 and 2005 and underwent periodic surveillance or received treatment in Anyang Tumor Hospital, Henan Province, China (Paper II).

Diagnostic tests and surgical or oncological treatments were performed depending on the clinical symptoms and on the stage of advancement of the disease, consistent with the conventional rules of therapy of esophageal cancer, including ongoing clinical trials and accepted palliative care.

Tissue samples of Barrett's esophagus and dysplasia were obtained from patients who underwent diagnostic tests as a result of increasing symptoms of dysphagia or a local irritation in the esophagus. Chest and esophageal x-rays with contrast, blood tests and endoscopy with a subsequent pathological evaluation of tissue samples were performed at the Norwegian Radium Hospital, Rikshospitalet HF and at Anyang Tumor Hospital, depending on the samples' origin. The dysplasia samples were obtained by gastroscopy with a flexible gastroscope. They were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin.

Tissue samples of advanced esophageal carcinomas (SCC and AC) were obtained from nonoperated patients by gastroscopy (as a diagnostic test before choosing treatment) and from patients who underwent potentially curative resection of the esophagus (preceded by adequate diagnostic tests) in the above mentioned hospitals. Potentially curative resection was defined as removal of all gross tumor tissue, histologically confirmed absence of tumor tissue at the surgical margins and absence of distant metastases (Torzewski et al. 1997). The patients received either no treatment prior to surgery or underwent neo-adjuvant radiotherapy. The gastroscopies were carried out using a flexible gastroscope. Esophagectomies were performed through laparotomy and a right- or left-sided thoracotomy with an abdominal approach through the diaphragm. Subsequent reconstitution was completed mostly by means of esophagogastrostomy, using the gastric tube through the retrosternal route, with construction of a cervical anastomosis. The surgery samples underwent the same fixation procedures as the dysplasia samples and were embedded in paraffin as a single sample (Norwegian tissue material), or were stored as tissue arrays (Chinese tissue material); each array contained tissue samples from 37 to 70 different patients.

As control group, samples of normal esophageal epithelium were collected from Norwegian patients, primarily operated at the Department of Surgical Oncology of the Norwegian Radium Hospital, Rikshospitalet HF for esophageal cancer between 1993 and 1999. Tissue samples were taken from normally looking squamous epithelium, located >5 cm from the tumor site. These samples were prepared for investigation in the same way as the samples described before. No tumor tissue was observed in the obtained samples, either macroscopically or microscopically.

In accordance with the WHO (WHO 1990), the dysplasia samples were histologically categorized into three groups: low-, moderate- and high grade dysplasia. The WHO classification also divides SCCs and ACs into three groups: well-, moderately- and poorly differentiated. Clinical classification was completed using TNM staging according to UICC Global Cancer Control (UICC 2005).

Cell lines

Cells deriving from well-, moderately- and poorly differentiated esophageal SCC respectively (KYSE450, KYSE140 and KYSE70 cell lines), and cells from normal esophageal epithelium (HET1A) were used in our study. The cell lines were provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Antibodies

We used the following antibodies in our experiments:
- Rabbit polyclonal antibody, isotype IgG, directed against human FAP-α, purchased from Abcam, Cambridge, UK
- Monoclonal rat antibody (clone E26), directed against the 200-220 kDa form of DPPIV obtained from Prof. W-T Chen (Department of Medicine, State University of New York, Stony Brook, N.Y., USA)
- Anti-human dipeptidyl peptidase IV, polyclonal goat antibody, obtained from R&D Systems, Minneapolis, MN
- Rabbit polyclonal antibody, directed against human HMW-scuPA (54 kDa), HMW-tsuPA (52 kDa) and LMW-scuPA (33 kDa), purchased from Abcam, Cambridge, UK

Laboratory methods

Immunohistochemistry

Tissue samples were fixed in 4% buffered formalin, embedded in paraffin, cut at four microns, dried in the oven at 70 °C overnight before deparaffinization and rehydration through decreasing concentrations of alcohol to running tap water. The slides required no pre-treatment. The EnVision+ system from Dako Cytomation was used according to the kit manual, and haematoxylin was used for counterstaining. Appropriate

negative and positive controls were applied according to the antibodies' specificity (M&M, papers I-III).

Semi-quantitative analysis of immunohistochemistry

The seprase-, FAP-α-, DPPIV- and uPA expressions were semi-quantified using a visual grading system in which the staining intensity was categorized into four groups; 0, 1+, 2+, 3+, where group 0 was defined as having a complete absence of staining. Groups 1-3 were defined as groups with positive staining of increasing intensity as compared to the positive control. The number of positive cells was also categorized into four groups, where group 0 was defined as total absence of positive cells, and groups 1-3 were defined as groups with positive cells counted in percentages; 0 = 0%, 1 = <25%, 2 = 25-50%, 3 = >50%. The outcome was calculated by multiplying the corresponding values from staining intensity by the number of positive cells and was subsequently divided into four final groups: 0, 1+, 2+ and 3+.

In order to guarantee quality control, two independent pathologists performed the sample interpretation separately. Diverging cases were discussed until an agreement was reached.

Immunoblotting

Cells from cell lines were lysed in cold TGH buffer (1% Triton X-100, 10% glycerol, 20mM Hepes, pH 7.2, 100mM NaCl) containing 1mM phenylmethylsulfonyl fluoride, 10μg/ml leupeptin and 1mM Na3VO4. After shaking, the lysate was incubated for 60 min at 4 ^{o}C with rocking. Insoluble materials were removed by centrifugation (14.000 rpm x g, 20 min) at 4 ^{o}C, and the supernatant was collected. Total protein concentration in each sample was estimated with the Bradford analysis (Bio-Rad). SDS-PAGE was performed in 12% gels as described by Laemmli (Laemmli 1970), and the proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL, Millipore, Bedford, MA). After one h blocking with 5% non-fat dry milk powder in TBS (TRIS Buffered Saline consisted of 137 mM NaCl, 25 mM TRIS and 2.7 mM KCL, pH 7.6) containing 0.05% Tween-20, the membranes were incubated at 4 ^{o}C overnight with tested antibodies at adequate dilution. The membranes were then washed three times for 10 min with PBS (Phosphate Buffered Saline consisting of 137 mM NaCl, 12 mM phosphate and 2.7 mM KCL, pH 7.6)/0.1% Tween-20 and finally incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for one h at room temperature. The membranes were then again washed three times for 10 min with TBS/0.05% Tween-20. A chemiluminescent detection reagent (ECL Plus, Western blotting detection system, GE Healthcare) was used for peroxidase signal detection. To ensure equal loading of proteins, the same membranes were reprobed with rabbit polyclonal anti ERK-2 (SC-154) antibody (Santa Cruz Biotechnology, CA, USA) at dilution 1:50000 in 5% milk.

Quantitative real-time RT-PCR analysis

Total cellular RNA was extracted from cell lines (KYSE450, KYSE140, KYSE70 and HET1A) using the TRIZOL reagent (Invitrogen, Carlsbad, CA). The high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used to reverse-transcribe obtained RNA (0.8 μg) in a 20 μl reaction mixture using random

primers. The real-time PCR analyses were performed using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays for FAP-α, DPPIV, uPA and GUS (Applied Biosystems). A total of 5 µl cDNA, diluted at 1:10, was used in 25 µl PCR mixtures with 900 nM of each primer and 250 nM TaqMan probe. The reactions were carried out in a 7900HT Fast Real-time PCR system (Applied Biosystems) with the following program: 95 ^{o}C for 20 s followed by 40 cycles of 95 ^{o}C for one s, 60 ^{o}C for 20 s. Each sample was run in triplicate. The threshold cycle (CT) values of the amplification reactions were determined automatically using RQ Manager 1.2 software (Applied Biosystems). The FAP-α, DPPIV and uPA relative mRNA expression level was normalized with respect to the beta-glucuronidase (GUS) gene, which had stable transcript levels under these experimental conditions. The tumor / normal ratio of the normalized target transcript expression was calculated by means of the $2^{-\Delta\Delta C}$ method (Livak & Schmittgen 2001) from three independent experiments.

Enzymatic activity biochemical assay

For enzymatic activity assays, near-confluent cells from KYSE450, KYSE140, KYSE70 and HET1A cell lines were incubated for 18 h in fresh medium (RPMI 1640, Invitrogen) containing 1% FCS. Then, media were collected and cells were lysed in lysis buffer (0.1 M NaCl, 10 mM Na2HPO4, 1% Triton X-100, 1 mM EDTA). The DPPIV-like activity was assayed by measuring the cleavage of Gly-Pro-NH-Np substitute (Gly-Pro-4-nitroaniline, Sigma) at pH 8.0 by either cell lysate or medium collected from cells after 18 h incubation, according to the standard procedure provided by Sigma. Twenty-five µl of sample (medium or lysate) were incubated in 0.1 M Tris (pH 8.0) with 0.5 mM Gly-Pro-NH-Np in a total volume of 200 µl at 37 °C for different time points in flat-bottom 96-well microplates. Activity was determined by measuring absorption at 405 nm with Biotrak Microplate Reader (Amersham). uPA activity was measured in an analogous way (using the chromogenic substrate) with the CHEMICON uPA Activity Assay Kit. Total protein concentration in samples was assayed by the Bradford method (Bradford 1976).

Statistical analysis

Associations between variables were assessed using Chi-square tests (Pearson and linear-by-linear association). Differences between quantitative variables in independent groups were tested by Mann-Whitney tests. They were all two-sided tests. Comparison of survival between the groups was performed using log-rank tests. Survival curves were calculated with the Kaplan-Meier product-limit method. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 13.0 and 15.0 for Windows.

6. SUMMARY OF PAPERS

PAPER I

DPPIV is a transmembrane serine protease involved in the process of tumor invasion and development of metastasis in human cancers. In this article we investigated the expression of DPPIV in cancer and stromal cells of both esophageal AC and SCC. We analyzed tissue material from 159 patients treated in our hospital between January 1987 and November 2002 (SCC (n=90) and AC (n=69)) using immunohistochemistry. The patients were divided into two groups to obtain more specific results: irradiated and non-irradiated patients (n=46 and n=113 respectively).

We also performed Western blotting on SCCs and normal esophageal epithelium cell lines, as well as on fresh frozen tissues from ACs and normal esophageal epithelium. Results of immunostaining were compared with the patients' clinicopathological features.

Immunohistochemistry of the whole population revealed differences between DPPIV expression in AC and SCC cells: DPPIV expression was higher in ACs than in SCCs. In contrast, DPPIV expression in stromal cells was higher in SCC stroma than in AC stroma. In the whole population, DPPIV was also significantly associated with lymph node metastases, distant metastases and age. The expression pattern revealed that a high expression of DPPIV involved a higher risk of distant- and lymph node metastases. The correlation between DPPIV expression and age was proportionally inverted: carcinoma cells of older patients contained a lower level of protease.

The separate analyses of SCC and AC showed a correlation between the expression of DPPIV in cancer cells and distant metastases only in the AC group. We did not find any associations between DPPIV expression in cancer cells and clinicopathological features in SCCs. Further analyses of the two groups showed no associations between DPPIV expression in stromal cells and clinicopathological features. Radiotherapy had no impact on DPPIV expression in the analyzed tissue samples. There was no correlation between DPPIV expression in cancer or stromal cells and survival of the patients.

Immunoblotting did not show any detectable DPPIV in the cell line originating from normal esophageal epithelium or fresh-frozen tissue samples of normal esophageal epithelium. In contrast, DPPIV was present in all carcinoma samples. SCC cell lines showed a decreasing intensity of DPPIV from well to poorly differentiated carcinomas. In the fresh-frozen cancer tissue samples, the highest DPPIV expression was detected in moderately differentiated carcinomas. Poorly and well differentiated carcinomas showed a lower expression of DPPIV.

Thus, differences in the DPPIV level in esophageal carcinomas compared with normal epithelium showed that esophageal malignancies were associated with an increased amount of cell surface-bound DPPIV.

PAPER II

In an attempt to elucidate the role of the serine proteases seprase, DPPIV and uPA in the degradation of the ECM and in the progression of esophageal SCC, we studied the expression of the proteases in esophageal dysplasia, invasive carcinomas and normal epithelium by using immunohistochemistry. Tissue samples from different stages of dysplasia (n=85) and from advanced SCCs at different stages (n=144) were obtained from patients treated at Anyang Tumor Hospital, Henan Province in China. Additionally, we performed real-time RT-PCR, Western blotting and enzymatic activity biochemical assays on cell lines originating from different SCC grades and from normal esophageal epithelium.

Seprase, DPPIV and uPA immunoreactivity were demonstrated in dysplastic and cancer cells as well as in stromal cells adjacent to dysplasia and cancer sites, but not in normal epithelium. A significant association between uPA expression and sex, tumor size and histological classification in carcinomas was shown. Increased expression of DPPIV in cancer cells correlated with longer patient survival. Furthermore, no significant associations between seprase and clinicopathological features either in dysplasia or in carcinomas could be found. Finally, using Western blotting, real-time RT-PCR and enzymatic activity assays, we demonstrated higher levels of the proteases in SCC cell lines than in normal esophageal epithelial cell lines.

These results showed that seprase, DPPIV and uPA were expressed in both premalignant and malignant forms of SCC, but lacking in normal esophageal epithelium, confirming thus their involvement in the SCC neoplastic progression. This fact indicates the possibility that these serine proteases play a role in the transformation from normal to dysplastic epithelium.

PAPER III

We examined FAP-α and uPA expression in premalignant and malignant stages of esophageal AC by immunohistochemistry. The findings were compared with clinicopathological features. Tissue samples of Barrett's esophagus with and without dysplastic changes were obtained from 22 patients, whereas tissue samples of irradiated and non-irradiated ACs of different histological grades were collected from 69 patients. We also performed Western blotting on fresh-frozen tissue samples obtained from both carcinoma tissue and normal esophageal epithelium.

There were no significant associations between the protein expressions in metaplastic and dysplastic cells and the histological grading or development of cancer. In contrast, stromal uPA expression was significantly higher in metaplastic and low- / moderate grade dysplasia than in high grade dysplasia. No such association could be found for FAP-α.

We did not find any correlations between FAP-α and uPA expression and clinicopathological features in carcinoma cells. Division of AC patients into two subgroups, irradiated patients (n=18) and non-irradiated patients (n=51), did not reveal any changes in the analyses.

Examination of the stromal protease expression on the other hand, exhibited significant correlations between FAP-α and depth of tumor invasion, as well as uPA and lymph node metastasis. High stromal FAP-α expression was detected in T1 tumors, compared to a low FAP-α activity in T2-T4 tumors. Stromal uPA overexpression was found in tissue samples of patients without lymph node metastases, while low uPA expression was associated with lymph node metastases.

The analysis of patients relating to radiotherapy revealed that non-irradiated patients had a higher stromal FAP-α concentration than irradiated patients.

We did not find any correlation between FAP-α or uPA expression and survival of the patients, but due to the modest number of patients, a type II error can not be excluded.

Immunoblotting showed higher protease expression in carcinoma tissues than in normal esophageal epithelium.

These results suggest that FAP-α and uPA expression in metaplastic, dysplastic and esophageal cancer tissue, including stroma adjacent to cancer sites, is associated with the early neoplastic progression of esophageal lesions.

PAPER IV

Esophageal cancer is not a common cancer in Norway, but remains a problem due to low survival rates and considerable treatment morbidity. We analyzed and compared clinical and pathological data of patients treated at the Norwegian Radium Hospital**,** Rikshospitalet HF, Oslo, during the last two decades (1988-1997 and 1998-2007). During the last 20 years, we observed a significant change in the occurrence of esophageal SCCs in relation to ACs in the Norwegian population. The AC incidence has increased from 5-10% to more than 50% in the last decade, while the incidence of SCCs has decreased. Additionally, a change in the pattern has also been observed: ACs occur in younger patients and are primarily related to GERD.

An important aspect of this two-decade study was to evaluate the general impact of anti-reflux therapy and surveillance of premalignant lesions and if this could be reflected in tumor stage, patient demographics and treatment results. This was not a randomized study but it gave us a possibility to get an impression of the effect of preoperative radiation on some biological markers and on the final treatment outcome, survival and recurrence rates. Based on the observations of our patients, we concluded that no significantly improved survival could be demonstrated from preoperative irradiation, but a trend towards improvement was seen. Finally, we found that the change from SCC to AC did not lead to improved long time survival in our patients.

Our results allowed us to observe that the most important epidemiological difference between SCC and AC is the strong association between GERD and AC. Furthermore, Barrett's esophagus was clearly associated with AC in more than one-fourth of the cases and probably in a lot more due to the fact that small Barrett's lesions are often completely supplanted by tumor tissue. Almost all patients suffered from dysphagia or dysphagia-related symptoms at the time of diagnosis, demonstrating that even for AC, usually in the distal esophagus, this is a late symptom. A very modest increase in early cancer lesions referred in the last decade demonstrates that an increased vigilance of GERD-induced lesions may be of importance for decisive local treatment instead of esophagectomy following dysphagia diagnosed advanced tumors.

In conclusion, upcoming studies should focus on developing strategies for early detection of the disease, using specific markers appearing in precancerous stages, separating thus completely or partially premalignant from malignant stages.

7. GENERAL DISCUSSION

The serine proteases' ability to degrade the ECM is considered to be a prerequisite for invasion and spreading of cancer cells (Duffy 1987, Zukker 1988, Birkedal-Hansen 1995, Hewitt & Danø 1996). Numerous factors are involved in this process, but the direct and indirect interaction between the proteases and the basement membrane seems to play a pivotal role in the initiation of tumor cell invasion (Duffy 1987).

Previous studies on serine proteases focused mainly on enzymes derived from tumor cells (Nishino et al. 1987, Torzewski et al. 1997, Kikkawa et al. 2004). Recently, however, enzymes produced by stromal cells bordering tumor sites have also been considered as a strong factor implicated in tumor progression (Iwasa et al. 2003, Mori et al. 2004). Although serine protease expression has been investigated in several carcinomas, there is still limited knowledge about their presence in esophageal malignancies.

Thanks to our collaboration with Anyang Tumor Hospital in China, we have access to a large series of esophageal carcinomas allowing us to study serine protease expression in premalignant and malignant stages of both SCC and AC. SCC, previously considered a dominant form of esophageal cancer worldwide, shows today a slightly decreasing incidence in the Western world, while it remains the major esophageal carcinoma type in the developing countries (Devesa et al. 1998, Blot & McLaughlin 1999). The SCC occurrence in certain areas of Asia or Africa is so high that screening programs could be considered a method for detecting premalignant and early stages of carcinoma, trying thus to reduce mortality among specific populations (Sammon 1998, Saidi 1999, Wang et al. 2002, Yang et al. 2002,). The Western world, on the other hand, suffers from increasing numbers of AC, whose association with GERD and Barrett's esophagus is commonly known. The latter could be considered a premalignant stage and by many suggested to undergo strict surveillance (Blot & McLaughlin 1999, Lagergren et al. 1999). The long-time effects on survival from esophageal cancer of such surveillance regimens have so far not been convincingly demonstrated.

We still believe that monitoring precancerous stages is important for early detection and local intervention and thus reducing mortality and morbidity. No blood test has yet been found which could distinguish premalignant from malignant stages. Histological biopsies and morphological evaluation still remain the major diagnostic methods (Dong et al. 2002).

In the current study, we focused on the analysis of three interesting indicators (seprase / FAP-α, DPPIV and uPA) and their possible role in the evolution of premalignant and malignant stages of both major types of esophageal carcinoma, SCC and AC. We compared their expression levels with clinicopathological features in a series of patients, using the clinical databases of the two previously mentioned cancer hospitals (papers I-III).

The analysis of clinical information related to Norwegian patients (Paper IV) has given a substantial insight into the rapidly changing esophageal carcinoma epidemiology in Norway and our diagnostic and treatment methods over the last two decades. Both pathological and clinical studies contributed to a better understanding of the evolution and differences between the two types of esophageal carcinomas and their premalignant stages. Results obtained from histopathological and biochemical studies pointed to the identification of possible markers that could be used in future clinical work to characterize precancerous stages as well as invasive cancers.

Main findings

Main findings concerning the expression of serine proteases in the analyzed tissue samples:

- Seprase, DPPIV and uPA immunoreactivity was found in dysplastic and SCC cells as well as in stromal cells adjacent to dysplasia and cancer sites, but not in normal epithelium (Paper II).
  - *A significant association between uPA expression in carcinoma cells and sex, tumor size and histological classification in SCCs was found.*
  - *High expression of DPPIV in carcinoma cells correlated with longer survival of the patients with SCC.*
  - *No significant associations between seprase expression and clinicopathological features either in dysplasia or in carcinomas were found.*
  - *SCC cell lines had higher levels of seprase, DPPIV and uPA than normal esophageal epithelial cell lines.*

- DPPIV displayed a significantly higher level in ACs as compared to SCCs, while no DPPIV was detected in normal esophageal epithelium (Paper I).
  - *Cellular overexpression of DPPIV in patients with AC was associated with distant metastases.*
  - *Radiotherapy in patients with SCC or AC had no impact on DPPIV expression in the analyzed tissue samples.*

- Seprase and uPA were detected in metaplastic-, dysplastic- and AC cells, as well as in stroma bordering metaplasia-, dysplasia- and carcinoma sites (Paper III).
  - *Stromal seprase expression was associated with depth of tumor invasion, while stromal uPA expression correlated with lymph node metastases in AC.*
  - *Stromal uPA expression in cells with dysplasia correlated with histological grading.*
  - *Higher protease expression in AC cells than in normal esophageal epithelium was found.*

Main findings concerning the analyses of the clinicopathological material from referred patients (Paper IV):

- Esophageal cancer showed an increased occurrence of AC in the distal part of the esophagus and, in the last two decades, was frequently associated with Barrett's esophagus in the Norwegian population.

- No significantly improved survival of the patients could be demonstrated from preoperative irradiation, but a trend towards improvement was seen.
- The change from SCC to AC did not lead to improved long time survival of the patients.

Serine proteases in dysplastic-, cancer- and stromal cells of SCC and AC

When analyzing tissue samples of different stages of dysplasia evolving into SCC (Paper II), we detected the strongest serine protease immunostaining in grade III dysplastic cells (carcinoma *in situ*) and the weakest in grade I, which turned out to be a common pattern for all three enzymes. The same staining pattern was also found in stromal cells adjacent to dysplasia sites, while normal epithelium did not show any staining at all. Considering the absence of serine protease expression in normal epithelium, the point of transformation from normal to dysplastic epithelium may be crucial for the activation of serine proteases, and, subsequently, their extracellular and pericellular proteolytic activities. Furthermore, the activity, or most likely, the production of serine proteases in both dysplastic cells and stromal cells adjacent to dysplasia increased in parallel to the advancement of dysplasia, showing a clear, positive association between the serine protease level and the degree of premalignancy. Finding serine proteases in premalignant and stromal cells indicates a close relationship between dysplastic and stromal cells, where dysplastic cells possibly stimulate stromal cells to generate e.g. serine protease activity. In relation to this and to what will be discussed further below, we thus concluded that serine proteases contribute to all stages of both premalignant and malignant progression in SCCs, and not only to the latter, as was previously suggested (López-Otín & Matrisian 2007).

On the other hand, when focusing on the expression of seprase and uPA in Barrett's metaplasia and dysplasia, eventually developing into AC (Paper III), no apparent association between protease expression and dysplasia stage could be identified, although the presence of proteases was evident in both dysplastic and stromal cells. Like in the case of SCCs, this finding too points towards an interplay between premalignant and stromal cells in this kind of carcinoma. It is noteworthy that this common trait concerned two histologically different premalignant conditions. With regards to this finding, we believe that the transmembrane proteases exist as signaling molecules on dysplastic cells and stromal cells adjacent to dysplasia in precancerous stages of both SCC and AC, probably interacting with other molecular factors exhibiting enzymatic activity, contributing to the development of invasive carcinomas.

The common immunostaining pattern for all tested proteases observed in dysplastic and stromal cells (Paper II) supported former studies, indicating the presence of supramolecular complexes such as seprase-uPA, seprase-DPPIV or even seprase-DPPIV-uPA on the cell membrane (Fig. 3) (Scanlan et al. 1994, Ghersi et al. 2002 & 2006, Chen & Kelly 2003, Kelly 2005). Although we did not specifically investigate the presence of such complexes in dysplastic or stromal cells, the similar enzymatic

activity of all three proteases detected in DPPIV-like and uPA enzymatic activity assays performed on cell lines reinforced the hypothesis about serine protease complexes.

Analysis of DPPIV alone in carcinoma cells (Paper I) showed that DPPIV expression was significantly higher in ACs than in SCCs, indicating that tumors originating from histologically different epithelial types may influence the production or expression of protease to a different degree. We presume that metaplasia occurring in the distal esophagus, where columnar cells replace stratified epithelium, and where dysplasia and AC frequently develop, significantly enhances DPPIV production in affected cells. This assumption is in agreement with Chaves' study (Chaves et al. 1999) in which she detected DPPIV in Barrett's esophagus and AC related to it, thus showing a direct association between these two conditions. Enhanced protease production may even be a necessary condition for the metaplastic process *per se*. The lower level of DPPIV found in SCCs in our study does not lessen the proteases' role in the development of this carcinoma type, but indicates that, in addition to DPPIV, other cell-surface-associated proteolytic enzymes could strongly be involved in the growth of SCCs, or, that SCCs require less DPPIV activity compared to ACs in order to develop equal invasive potential.

Furthermore, we found that DPPIV was also present in stromal cells bordering cancer sites (Paper I), similarly to previously mentioned findings in dysplastic changes. Stromal DPPIV presence in SCC, as well as in AC tissue samples, may indicate that cancer cells of histologically different carcinoma types can even influence adjacent stromal cells and initiate an additional stromal production of proteases. This finding is similar to other studies showing the presence of the DPPIV homologue, seprase, in fibroblasts, macrophages and endothelial cells adjacent to tumor sites in human gastric and colorectal cancer, where stromal seprase expression was associated with lymph node and liver metastases (Iwasa et al. 2003, Okada et al. 2003, Mori et al. 2004). Our results indicate that stromal DPPIV (no association between stromal DPPIV and clinicopathological features) is probably not directly, or only modestly responsible, for ECM degradation in esophageal carcinomas. It could rather be a useful activator of other enzymes, i.e. metalloproteinases or DPPIV-associated serine proteases which serve as workhorses.

Investigation of seprase, DPPIV and uPA occurrence in SCCs (Paper II) and of FAP-α and uPA in ACs (Paper III) showed again expression of the serine proteases not only in carcinoma cells but also in neighboring stromal cells. The immunoreactivity of uPA and DPPIV in SCC cells was characterized by a decreasing intensity pattern from well differentiated towards poorly differentiated carcinomas. As for seprase, although an expression of more than 93% was observed in SCC samples, we could not demonstrate any distinct expression pattern. AC cells, in contrast, despite abundant FAP-α and uPA presence, showed no correlation between serine protease expression and carcinoma advancement (Paper III). Interestingly, stromal FAP-α and uPA expression was inversely correlated to T and N and, in consequence, to tumor progression in general.

We suggested previously that cancer cells are able to stimulate the production of proteases and other enzymes essential to ECM degradation in stromal cells, which is consistent with other studies (Sawhney et al. 1992, Iwasa et al. 2003). The stromal serine protease expression could also originate from serine proteases which are mainly produced by cancer cells and may attach to stroma during their activation (Chen et al. 2006). Regardless of the cause of its expression, stromal protease expression denotes that stroma is able to partly assume control over the serine protease production in advanced cancer stages, possibly as a result of a reduced serine protease production in highly undifferentiated carcinoma cells. Intensification of such stromal behavior may be dependent on the carcinoma histology. For example, the fact that DPPIV and uPA expression in SCC cells decreases, whereas tumor aggressiveness increases, clearly illustrates this aspect (Paper II). A question arises at this point: do stromal cells take over the serine protease production from poorly differentiated carcinoma cells in SCCs and thus act as a major factor contributing to tumor invasion in highly invasive carcinomas?

As for ACs, lower stromal FAP-α and uPA expression positively correlated with tumor invasion and lymph node metastases, indicating that AC cells still possess more control than stromal cells concerning the serine protease production. This hypothesis is supported by results where DPPIV expression in stromal cells was greater in SCCs than in ACs and in cancer cells lower in SCCs than in ACs (Paper I). We showed thus an obvious difference in the serine proteases' biological behavior in two histologically different esophageal carcinomas. This conclusion derives from analyses of both carcinoma cells and stromal cells adjacent to cancer sites, the latter seeming to play a more important role in neoplastic progression than was previously assumed (López-Otín & Matrisian 2007).

Considering the fact that the analyzed serine proteases all belong to one family, that DPPIV and seprase share nearly 50% of sequence identity and that the enzymatic activity assay results performed on esophageal carcinoma cell lines and normal esophageal epithelium were similar for all three enzymes, we presume that the proteases operate in an analogous way regarding ECM degradation. Altogether, these findings point to a complex interaction between the serine proteases themselves and between premalignant, malignant and stromal cells in the development of neoplastic process.

The serine protease involvement in tumor progression appears to be evident. Previous studies presented them mostly as tumor promoters (Hewin et al. 1996, Cheng et al. 1998, Iwasa et al. 2003). However, contradictory findings concerning DPPIV, exposing it as a tumor suppressor, were also reported (Wesley et al. 1999, Pethiyagoda et al. 2000, Kikkawa et al. 2005), whereas seprase and uPA were only associated with an increased tumorigenicity. In our study, we observed that high uPA and DPPIV expressions in SCC cells correlated with a better prognosis (Paper II), which corresponds to the tumor suppressor hypothesis. However, this finding has to be interpreted with caution, as we cannot exclude the possibility that even stromal serine protease in carcinomas may significantly contribute to preserving their malignancy and thus tumor development. In such a case, it is important that not only

malignant but also stromal cells adjacent to lesions be analyzed for protease expression.

In our study we have analyzed three proteins considered to be important in tumor invasion and as prognostic markers. However, many other molecular factors are involved in neoplastic progression. Recently, many potential predictive and / or prognostic markers for esophageal carcinoma have been identified (Vallböhmer & Lenz 2006). For example, the p53 molecule, with major functions in regulating apoptosis and in G_1-S cell cycle transition, is early and often mutated in SCCs and ACs (Shimada et al. 2000). Rb and p16, central regulators of the G_1 cell cycle check point, are also altered in both histological esophageal carcinoma subtypes (Roncalli et al. 1998, Sturm et al. 2001). Human epidermal growth factor receptor 1 seems to be a reliable prognostic and predictive marker in esophageal cancer patients (Mendelsohn & Baselga 2000 & 2006, Miyazano et al. 2004). Alterations of other markers, such as angiogenetic (b-FGF, TGF-α, TGF-β, VEGF and Cox-2) or apoptotic (Bax) markers are frequently associated with the overall outcome of SCCs and ACs (Inoue et al. 1997, Shih et al. 2000, Aloia et al. 2001, Ikeguchi et al. 2001, Sturm et al. 2001, Shimada et al. 2001, Ogata et al. 2003, Fukai et al. 2003, Kuo et al. 2003, Kleespies et al. 2004, Han et al. 2005). As for the transmembrane protease family, MMP-2, MMP-7 and MMP-9 were studied in esophageal cancer and found to be negative prognostic indicators (Tanioka et al. 2003, Ishibashi et al. 2004, Sharma et al. 2004). Thus, despite the indisputable biological importance, the value of these and several other investigated molecules as clinical biomarkers for prognosis, prediction and early detection is still under evaluation.

Clinical study interpretation

The trend in the incidence rates of esophageal cancer generally increased in the last two decades in many Western countries. While rates for esophageal SCC remained stable or increased slightly, occurrence of AC in the distal esophagus augmented significantly. At the time of diagnosis, most esophageal cancer patients already suffer metastatic disease (Layke & Lopez 2006, Stoner et al. 2007). This results in a poor prognosis: only one of five esophageal cancer patients survives more than three years after initial diagnosis (Younes et al. 2002, Polednak 2003, Stoner et al. 2007).

We observed a distinct trend of increasing esophageal AC and decreasing SCC in the referred population during the last 20 years. In order to evaluate the rate, risk factors, advancement and treatment of both esophageal carcinomas, we analyzed the clinical data and cancer tissue samples of 347 patients treated in our hospital between 1988 and 2007. We wanted to know if the change in carcinoma type influenced survival from esophageal cancer in the last two decades.

Our observations confirmed the previously mentioned tendency of increasing esophageal AC and slightly decreasing SCC. This is consistent with other studies in Europe and in the USA (Devesa et al. 1998, El-Serag et al. 2002, Brown et al. 2008). We found that over one-fourth of patients with AC reported a Barrett's esophagus

history, mostly connected to GERD symptoms. Also, AC was diagnosed more frequently among young patients (30-55 years) (Paper IV). As for SCC, tobacco and alcohol were the two most recognized risk factors. This significant change in tumor type seems to be strongly related to changes in Norwegian lifestyle. In the last decades, the total number of smokers constantly diminished, whereas increasingly more Norwegians suffer from obesity (FHI 2004 & 2007, SSB 2008). Weight gain has been postulated to enhance gastroesophageal reflux, which can lead to a sequence of changes from Barrett's metaplasia to dysplasia and cancer.

In Norway, treatment of GERD has been taken seriously, and considerable resources have been allocated to this problem. If the diagnosis is made, treatment with proton pump inhibitors (PPI) is refunded by the health authorities. Endoscopy and regular follow-up with biopsies of Barrett's changes and dysplasia are recommended as well as surgery when drug therapy does not alleviate symptoms or mucosal changes develop further. However, despite these efforts, while surgical and medical treatment of the symptoms is generally available and almost free of charge, we could not demonstrate any significant improvement, neither in the survival of esophageal cancer in general or in the subgroup of AC.

Identification of genetic changes within families with increasing cancer incidence is important, and diagnostic methods should focus on the development of strategies for early detection of neoplastic malformations, using specific markers emerging in precancerous stages. This could give us information about the disease advancement and thus help us to determine an appropriate local treatment.

Since we do not yet have any specific molecular markers characteristic for esophageal carcinoma, we should focus on the serine proteases which seemed to play an important role in the progress of esophageal malignancies. The detection of both FAP-α and uPA in precancerous stages of AC (Paper III) confirmed previous results describing the involvement of serine proteases in neoplastic progression from its very beginning and their proteolytic activity already in full progress in metaplastic and dysplastic cells. Interestingly, we also detected expression of seprase, DPPIV and uPA in stromal tissue adjacent to dysplastic sites. The staining intensity in stroma increased in parallel with the changes in dysplastic cells, with strongest stromal staining in grade III dysplasia (Paper II). This finding corroborates a coherence between stromal and dysplastic cells in cancer evolution. Here, the question is rising: is it possible to keep precancerous stages under surveillance by monitoring the molecular marker levels in biopsies? An affirmative answer should be formulated carefully, as much more research is needed to define to which degree the proteases are involved in the evolution of precancerous stages.

8. CONCLUDING REMARKS AND PERSPECTIVES

The low survival rate of esophageal cancer has not been improved during the last decade.

ACs have replaced SCCs as the most common type of esophageal cancer being treated with curative intention. This has not improved the low long-time survival rate from esophageal cancer, neither in our reference hospital nor in the national population. Improved neo-adjuvant regimens seem to be able to improve the survival rate significantly, but not to a large extent and with a considerably increase in cost and morbidity. Without a change towards early diagnosis, most patients will be candidates for palliative treatment. Our clinical results demonstrate that, as long as dysphagia and dysphagia-related symptoms are the diagnostic starting points, this will more or less continue to be the case.

We firmly believe that early diagnosis, combined with local treatment regimens, is the best strategy for improvement. The rapid increase in ACs in the Western world population is closely connected to symptomatic reflux and a slow development of precancerous lesions. Thus, aggressive treatment of reflux, either by means of anti-reflux surgery or efficient drug treatment, is mandatory. Dysplastic lesions are able to continue their development towards cancer, even after efficient anti-reflux therapy. Strict surveillance of such lesions by morphological and biological parameters is essential and should lead to local treatment of areas demonstrating development towards cancer or severe dysplasia. An efficient combination of biological markers is still elusive. The membrane proteins studied in this thesis have given us new knowledge of esophageal cancer development, but alone, they do not have the ability to detect lesions developing towards cancer and would have to be combined with other markers. The endeavour towards the most efficient combination of new and already characterized markers will have to continue.

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