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HEAD AND NECK CANCER – FACTORS AFFECTING TUMOUR GROWTH

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ABSTRACT

Head and neck cancer is the fifth most common cancer worldwide with an estimated annual global incidence of over 500 000 cases. These malignant tumours develop in the mucosal linings of the upper respiratory tract or in the salivary glands. The most common sites are in the oral cavity and larynx. Treatment modalities comprising surgery and chemoradiotherapy have improved significantly during the last 20 years, but not the long-term survival of patients. The aim of this thesis was to study the different factors affecting tumour growth in head and neck cancer that may have clinical implications in the future. Factors involving apoptosis, cell cycle activity, inflammation, and enzyme activity were of special interest.

The results of the thesis indicate that patients with malignant salivary gland tumours having the lowest level of actively replicating cells have the best prognosis. The largest amount of replicating cells in tongue cancer specimens was found in the peripheral areas of tumour nests. Metallothionein, a protein that can hinder apoptosis, was found in excess in the same areas, whereas apoptosis activity was considerably lower. Taken together, these results indicate that the most aggressive cancer cells are found in the peripheral areas of tumours where apoptosis may be hindered.

The expression of the death receptor Fas was higher in tongue cancer specimens than in normal mucosa. The expression of this receptor was studied further in two cell lines established from oral cancers. When a low dose of cisplatin was added to cell cultures, the Fas expression was enhanced in both cell lines and, furthermore, the Fas-induced apoptosis was increased in one of the cell lines. The results show that a common chemotherapeutic drug given in a low, less toxic dose may enhance receptor-mediated apoptosis of cancer cells.

Malignant solid tumours are often distinguished by an increased proteolytic activity resulting in invasive growth, neo-angiogenesis, and metastases. This activity is conducted by enzymes that are secreted from tumour cells, or from normal cells in the tumour microenvironment. The regulation of enzyme secretion may be mediated by cytokines, small signalling molecules also present in cancer tissue. The results of this thesis show that two cytokines can synergistically induce enzyme secretion (matrix metalloproteinase-1 and -9) from oral cancer cells. Cytokine tumour necrosis factor-alpha and hepatocyte growth factor added alone to cell cultures strongly stimulated secretion of these enzymes. Thus, the tested cytokines, which are commonly secreted by fibroblasts and immune cells, may promote tumour growth.

This thesis has contributed to an increased understanding of factors affecting tumour growth in head and neck cancer. The upcoming cancer therapies will be based on the increasing knowledge of these and other aberrant cellular mechanisms that may vary between different cancer forms.

LIST OF ORIGINAL PUBLICATIONS

I. Tumour growth fraction and apoptosis in salivary gland acinic cell carcinomas. Prognostic implications of Ki-67 and bcl-2 expression and of in situ end labelling (TUNEL). Hellquist HB, Sundelin K, DiBacco A, Tytor M, Manzotti M, Viale G. J Pathol (1997);181:323-329

II. Metallothionein and Fas (CD95) are expressed in squamous cell carcinoma of the tongue. Sundelin K, Jadner M, Norberg-Spaak L, Davidsson Å, Hellquist HB. Eur J Cancer (1997);33(11): 1860-1864

III. Effects of cisplatin, interferon-alpha and 13-cis retinoic acid on the expression of Fas (CD95), intercellular adhesion molecule-1 (ICAM-1) and epidermal growth factor receptor (EGFR) in oral cancer cell lines. Sundelin K, Roberg K, Grénman R, Håkansson L. J Oral Pathol Oral Med (2007);36:177-183

IV. Effects of cytokines on matrix metalloproteinase expression in squamous cell carcinoma in vitro. Sundelin K, Roberg K, Grenman R, Håkansson L. Acta Otolaryngol (2005);125(7):765-773

CONTENTS

ABBREVIATIONS

Bcl-2	Anti-apoptotic protein found initially in B-cell lymphoma
CDDP	Cisplatin
CDK	Cyclin-dependent kinase
c-met	Hepatocyte growth factor receptor
DISC	Death inducing signalling complex
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
Fas/CD95	Fas-receptor
FasL	Fas ligand
FLIP	FLICE-inhibitory protein
HGF	Hepatocyte growth factor
HNSCC	Head and neck squamous cell carcinoma
ICAM-1	Intercellular adhesion molecule-1
IFN-α	Interferon-α
IL-6	Interleukin-6
IL-8	Interleukin-8
Ki-67	Protein expressed in replicating cells
mAb	Monoclonal antibody
MMP	Matrix metalloproteinase
MT	Metallothionein
NK	Natural killer
p53	Tumour suppressor gene
sIL-6R	Soluble interleukin-6 receptor
SCC	Squamous cell carcinoma
TAF	Tumour-associated fibroblast
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TIL	Tumour-infiltrating lymphocyte
TNF-α	Tumour necrosis factor-α
TUNEL	Detection method for DNA breaks
VEGF	Vascular endothelial growth factor
13-cisRA	13-cis retinoic acid

1 INTRODUCTION

1.1 CANCER IN THE HEAD AND NECK

Head and neck cancer is the fifth most common cancer worldwide, with an estimated annual global incidence of over 500,000 cases (Parkin et al 2001). According to the Swedish Cancer Registry of 2005, 1145 new cases of head and neck cancer were diagnosed, representing 2.2% of the total cancer incidence in Sweden. Cancer in the head or neck region includes the nasal cavity, sinuses, lip, mouth, salivary glands, throat, and larynx. The most common form of malignancy is squamous cell carcinoma, which develops in the mucosal linings of the upper respiratory and digestive tracts. This area is further divided into sub-entities of which the oral cavity (including lips) and larynx are the most common sites of cancer. Salivary gland lesions are a relatively rare and morphologically diverse group of neoplasm and about 80% are benign (Speight and Barrett 2002). The major histopathological types of salivary gland carcinomas comprise mucoepidermoid carcinoma, adenoid cystic carcinoma, acinic cell carcinoma and malignant mixed tumours. It is worth noting that even though the salivary gland malignancies comprise approximately 5% of cancers of the head and neck worldwide, the frequency in Sweden is twice as high according to the Swedish Cancer Registry 2005.

Early head and neck cancers (stage I and II) are highly curable by surgery or radiotherapy combined with chemotherapy. The choice of treatment is dictated by the anticipated functional and cosmetic results of treatment, and by the availability of the particular expertise required of the surgeon or radiation oncologist for the individual patient. More advanced cancers (stage III and IV) are commonly treated by a combination therapy of surgery and chemoradiotherapy. Still, despite significant improvements in head and neck cancer treatment, long-term survival of head and neck cancer patients has only moderately improved during the last 20 years (Forastiere et al 2001). Up to 40% of patients with squamous cell carcinoma in the head and neck region present with metastatic disease and survival is dependent on the disease stage (Lefebvre 2005).

1.2 MALIGNANT TRANSFORMATION

The process of cancer development has been addressed in innumerable studies documenting its molecular basis and proposing genetic progression models for various tumour types. It is now well established that an accumulation of genetic and epigenetic alterations forms the basis of the transformation from a normal cell to a cancer cell, and is referred to as the process of multi-step carcinogenesis (Fearon and Vogelstein 1990). Two key genes were identified in a review of the most common genetic alterations in the head and neck carcinogenesis, namely the p53 and p16 genes (Gollin 2001). Furthermore, chromosomal losses at 3p21, 9p21, 13q21 and 17p13, and gains at 3q26 and 11q13 are frequently detected in large panels of head and neck carcinomas (Gollin 2001). These genetic alterations may result in activation of proto-oncogenes and growth factor receptors, inactivation of tumour suppressors and uncontrolled cell proliferation (Perez-Ordonez et al 2006). Although the initial genetic mutations are critical, the ultimate success of the cancer cell depends on the acquisition of several of the following alterations: autocrine growth signals, insensitivity to anti-growth signals, angiogenesis, ability to evade apoptosis, capacity for invasion and metastasis, and unlimited replicative potential (Hanahan and Weinberg 2000). Several aspects of these general characteristics of cancerous lesion will be discussed in the present context of head and neck cancer.

1.3 APOPTOSIS

1.3.1 General considerations

Apoptosis (programmed cell death) is a physiological process of cell elimination, which is important for both maintenance of cellular homeostasis, cell proliferation and differentiation. Disturbances in the cell death process may lead to uncontrolled cell growth and result in tumour formation. Apoptosis is morphologically characterized by cytoskeletal and organelle disruption, cell shrinkage, membrane blebbing, chromatin condensation and fragmentation of the cell into small membrane bound apoptotic bodies, which are phagocytosed by macrophages or neighbouring cells (Kerr et al 1972).

The process of apoptosis is thought to occur in three distinct stages: initiation, effector phase and degradation (Kroemer et al 1995). The initiation phase is induced by a variety of damaging events, including DNA damage, hypoxia, withdrawal of nutrients, growth factors or hormones, and activation of death receptors (Thompson 1995). The pathways that regulate and initiate the downstream effects and degradation phases of apoptosis are less diverse and are shared by many cells undergoing apoptotic cell death.

Two principal apoptosis pathways have been recognized, both of which result in activation of the caspase cascade. Caspases are proteases that are synthesized as inactive precursors (pro-caspases) that must be activated by proteolysis, performed by self-cleavage (Nicholson and Thornberry 1997) or by other caspases (Cohen 1997, Salvesen and Dixit 1997). The extrinsic pathway is mediated by activation of transmembrane death receptors of the TNF (tumour necrosis factor) receptor superfamily leading to activation of caspase-8 (Figure 1, see also section 1.3.3).

The other principal death-signalling pathway, the intrinsic pathway, is induced by cellular stress and involves release of mitochondrial apoptogenic factors, such as cytochrome c, apoptosis-inducing factor (AIF), and Smac/Diablo (Wang 2001) (Figure 1). When released into the cytosol, cytochrome c forms together with, ATP, apoptosis protease-activating factor-1 (Apaf-1) and pro-caspase-9 a complex called the apoptosome, which triggers the activation of the caspase cascade (Tsujimoto and Shimizu 2000). Caspase activation results in degradation of intracellular constituents and ultimately cell death, apoptosis (Kitson et al 1996). Fragmentation of DNA is one of the degrading processes that may distinguish apoptosis.

The extrinsic and intrinsic pathways to apoptosis can act independently, but under certain circumstances the extrinsic, pathway must rely on the amplification of the death signal provided by the mitochondrial pathway to induce apoptosis (see further section 1.3.3) (Li et al 1998, Luo et al 1998). Since the caspases play an important role in execution phase of apoptosis, it is conceivable that low expression or dysregulation of caspase function might influence the apoptotic process and result in inappropriate cell proliferation. The inhibitors of apoptosis (IAPs) are a family of cytosolic proteins that block cell death by binding to and inhibiting the action of caspases (LaCasse et al 1998). They function downstream of both the intrinsic and extrinsic pathways of cell death (Holcik et al 2001).

1.3.2 p53

The tumour suppressor p53 is known as the “guardian of the genome” due to its ability to integrate many signals that control life and death (Vogelstein et al 2000). Activated by various types of cellular stress, including DNA damage and oncogenic stress, p53 initiates gene transcription that ultimately arrests proliferation and prevents establishment of genetically altered genes. The selective elimination of stressed or damaged cells by apoptosis protects the organism from development of cancers. p53 can mediate apoptosis by both transcription-dependent and transcription-independent mechanisms.

A number of apoptosis related genes of the Bcl-2 superfamily (Balint and Vousden 2001) and TNF receptor superfamily (Bates and Vousden 1999) are transcriptionally regulated by p53. Futhermore, p53 binding sites have been detected in promoter regions of pro-caspase-6 and Apaf-1 genes (reviewed in Bossi and Sacchi 2007), and cathepsin D (Wu et al 1998). p53 is also known to directly stimulate apoptosis in a transcription-independent manner, by up-regulation of receptors such as Fas (Balint and Vousden 2001), or by regulation of mitochondrial membrane permeabilisation (Vousden 2005) indicating induction of cytochrome c release from the mitochondria (Vousden 2006). Loss of p53 function occurs in a high proportion of human malignancies (Hollstein et al 1991) and in oral cancer, the frequency of p53 gene mutations is more than 60% (Sakai et al 1992). The prognostic value of p53 in head and neck cancer remains controversial (reviewed in Lothaire et al 2006).

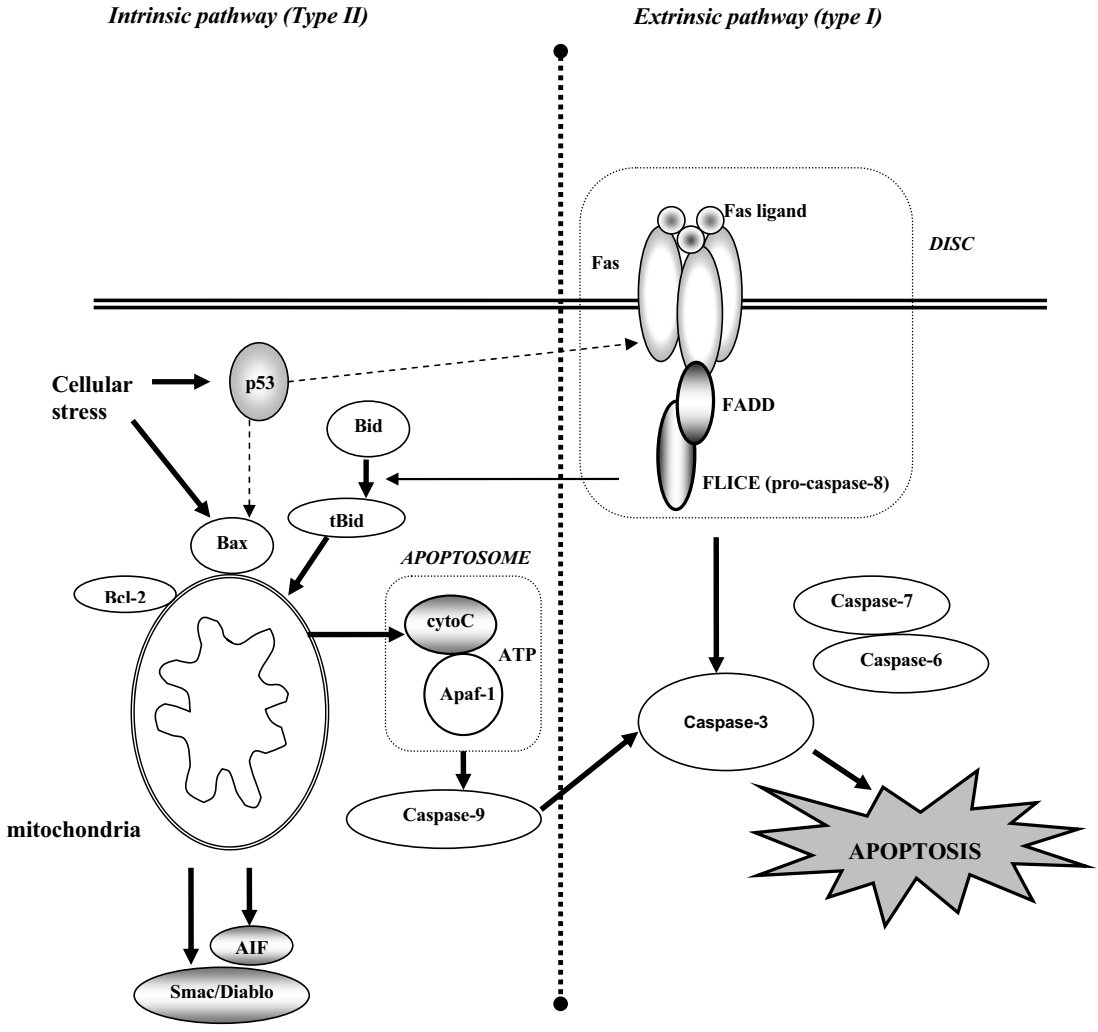

Figure 1. The apoptotic pathways. The extrinsic pathway is triggered by the activation of death receptor (Fas/CD95) upon its interaction with the ligand (FasL). Once activation occurs, death receptors form the "Death inducing Signalling complex" (DISC) that recruits, via the adaptor molecule Fas Associated Death Domain protein (FADD), multiple pro-caspase-8 molecules resulting in caspase-8 activation. This signal may be sufficient to trigger apoptosis (type I cells) but sometimes a caspase-8 mediated activation of Bid and mitochondrial pathway is needed for reinforcement of apoptosis signalling (type II cells). The intrinsic pathway is activated in response to extracellular stimuli (hypoxia, lack of nutrients, e.g.) and internal insults (DNA damage, e.g.). These diverse response pathways converge on the mitochondria altering the balance between pro-apoptotic (Bax/Bak) and anti-apoptotic (Bcl-2/BclXL) proteins. The dominance of pro-apoptotic proteins increases the mitochondrial membrane permeability resulting in release of the apoptogenic factor cytochrome c. Once in the cytoplasm cytochrome c forms the apoptosome with Apaf-1 (apoptosis protease-activating factor-1) and ATP, which activates caspase-9. Diablo/Second mitochondria-derived activator of caspase (Smac) inhibits the cytosolic inhibitor of apoptosis proteins (IAPs). The extrinsic and intrinsic pathways converge at the level of caspase-3 activation resulting in apoptosis.

1.3.3 Fas-receptor

The Fas-receptor, CD95, is a 45 kDa transmembrane death receptor expressed in both normal and tumour tissue (Trauth et al 1989, Owen-Schaub et al 1994). It belongs to the TNF-receptor super family that comprises almost 20 members, including the nerve growth factor receptor, CD27, CD30, CD40, OX40 (Smith et al 1994) and the death receptors TNFR1and TNFR2 (Milatovich et al 1991). The Fas ligand binds to the Fas-receptor leading to trimerization of the receptors. The death domains of the clustered intracellular receptors interact with the adaptor molecule FADD/MORT1, which is recruited to Fas upon activation (Kischkel et al 1995, Algeciras-Schimnich et al 2002) (Figure 1). Pro-caspase-8 (FLICE) also binds to the intracellular death domain of activated Fas and the formed complex is called the death-inducing signalling complex (DISC). In DISC caspase-8 molecules are brought in close proximity so that they can transactivate one another.

Two Fas signalling pathways (types I and II) have been identified in cell lines (Houston and O´Connell 2004). In type I, the induction of apoptosis is accompanied by production of large amounts of caspase-8, followed by rapid activation of caspases-3, -6, -7. Caspase-3 activation occurs before the loss of mitochondrial transmembrane potential, suggesting activation of a direct apoptosis cascade (Scaffidi et al 1998).

In type II signalling, there is a reduced DISC formation and activation of caspases occurs after loss of mitochondrial transmembrane potential (Scaffidi et al 1998). In type II signalling, caspase-8-cleaved Bid mediates release of mitochondrial apoptogenic factors that amplify the caspase cascade (Schendel et al 1999). Although many cancer cell lines express Fas death receptor on their plasma membrane, not all cell lines are sensitive to anti-Fas. FLIP (FLICE-inhibitory protein) is a dominant negative caspase-8 that suppresses the caspase cascade and apoptosis. Furthermore, intracellular sequestration of Fas receptor may cause resistance to apoptosis. Fas-associated phosphatase-1 can hamper Fas expression by binding to receptors in the cytosol and thereby inhibiting export of Fas to the cell surface (Ivanov et al 2003). Bennett et al (1998) showed that p53 activation caused redistribution of Fas from the Golgi complex to the cell membrane and induced apoptosis after incubation with a Fas-agonistic antibody CH-11.

Fas-mediated apoptosis is commonly involved in regulation of the immune system. The Fas-Fas ligand interaction plays a critical role in killing by cytotoxic T cell and negative regulation of several immune processes (Nagata and Golstein 1995). The receptor ligand (FasL) is found both in soluble and membrane bound form and is expressed on the surface of activated lymphocytes and NK cells (Nagata and Golstein, 1995). Gastman et al (1999) show expression of FasL on squamous cell carcinoma of the head and neck cell lines and on tumour cells in biopsy material. The highest expression for FasL was found closest to tumour-infiltrating lymphocytes and in tumours with substantial lymphocytic infiltrates.

1.3.4 The Bcl-2 protein family

Early evidence supporting the hypothesis that genes and proteins that play a role in tumorigenesis may be involved in the inhibition of cell death came from the observation that Bcl-2, which was originally cloned from the t(14;18) translocation breakpoint found in follicular B-cell lymphomas, was able to rescue lymphoid and myeloid cells from an otherwise inevitable death caused by withdrawal of the cytokine, interleukin-3 (Vaux et al 1988, Vaux et al 1992). Moreover, it was shown that mutations in *bcl-2* can, in turn, promote lymphomagenesis and influence the sensitivity of tumour cells to chemotherapy and radiotherapy.

At present, the Bcl-2 family of proteins comprises more than 30 members with either pro-apoptotic (e.g. Bax, Bid, Bak, Puma and Noxa) or anti-apoptotic functions (e.g. Bcl-2, Bcl-X_L and Mcl-1) (Cory et al 2003). The various members in the family show sequence similarities in the BH (Bcl-2 homology) domains and can form various types of homo- and heterodimers with each other. The proteins control apoptosis by regulating the release of apoptogenic factors from the mitochondria by a mechanism not yet fully resolved. Thus, high levels of the Bcl-2 protein will prevent apoptosis (Chiou et al 1994), while higher levels of Bax will have an opposite, pro-apoptotic effect (Korsmeyer 1995). It seems reasonable that the ratio between these pro- and anti-apoptotic proteins, rather than over-expression of one particular member, might influence tumour formation and/or the susceptibility of the tumour cells to undergo apoptosis. Interestingly, recent investigations show that some of the pro-apoptotic members of the Bcl-2 family (the BH3-only proteins Noxa and Puma) are regulated by p53 and may act as tumour suppressors (Oda et al 2000, Nakano and Vousden 2001). Bcl-2 overexpression has not been consequently associated with either poor or favourable prognosis in head and neck cancer. A better overall survival was reported for bcl-2-positive patients that were treated with radiotherapy compared to bcl-2-negative patients (Wilson et al 2001), although the opposite results were shown in a study by Gallo et al (1996).

1.3.5 Metallothionein

Metallothioneins (MT) are low molecular mass cystein-rich proteins with capacity to chelate several heavy metal ions (e.g. copper, silver, mercury, zinc and cadmium) (Theocharis et al 2004). These proteins regulate copper and zinc homeostasis and act as detoxification stations for heavy metals. Most adult mammalian tissues contain low basal levels of MT and they are mainly detected in the cytoplasm (Cherian et al 2003). However, a transient localization of MT into the nucleus in cell proliferation and differentiation has been reported (Apostolova and Cherian 2000). Four isoforms (MT-I, -II, -III and -IV) have been identified in mammals (Theocharis et al 2004) and they are inducible by several agents and conditions both *in vitro* and *in vivo*. The biosynthesis is primarily raised at the transcriptional level after, for example, injection or oral administration of heavy metals, exposure to inflammatory agents and cytokines as well as stress-producing conditions and hormones including glucocorticoids, glucagon and adrenalin (Kägi 1991). Thus, expression of MTs is suggested as a pro-survival factor. By exposing squamous cell carcinoma cell lines to IFN (interferon)-α-2a for 24 hours Gupta et al (1995) showed 30-40% higher levels of MT-II_A mRNA in IFN-α-2a treated cells compared to controls. Leyshon-Sorland et al (1993) showed nuclear distribution in human epithelial and fibroblast cell lines and a possible antagonistic role in tumour necrosis factor-induced cell death. The role of metallothionein in apoptosis has been further studied by Abdel-Mageed and Agrawal (1997), who showed that down-regulation of MT in breast adenocarcinoma cell lines with an 18-mer antisense phosphorothioate inhibited proliferation and also initiated apoptosis.

Thornalley and Vasak (1985) demonstrated that MTs are effective antioxidants able to quench free hydroxyl radicals and superoxide radicals due to their high content of cystein. This capacity to scavenge free radicals can protect tumour cells during radiotherapy (Thornalley and Vasak 1985). Many solid tumours have regions of hypoxia because of an impaired blood supply. When squamous carcinoma cells were subjected to 14 hours of hypoxia, significant accumulations of MT were found compared to aerobic controls (Murphy et al 1994). In the same study, high MT levels were maintained for up to 8 hours of reoxygenation. Metallothionein over-expression is also related to cisplatin resistance and poorer prognosis in squamous cell carcinoma of the esophagus (Hishikawa et al 1997). Studies on ovarian cancer cells show that only nuclear, not cytoplasmic MT expression is correlated with cisplatin resistance and poor clinical outcome (Surowiak et al 2007). Smith et al (2006) treated prostate cancer cells with zinc and demonstrated activation of MT gene expression and enhanced protein

expression. As could be expected, these cells showed a significantly decreased sensitivity to cisplatin and radiotherapy compared to controls. In a study including over 1000 patients with primary cutaneous melanoma, MT overexpression was a highly significant and independent factor for disease progression and reduced survival. (Weinlich et al 2006). However, the expression of MT is not universal to all tumours and the biological functions of MT during tumorigenesis need to be further elucidated.

1.4 CELL PROLIFERATION AND TUMOUR PROGRESS

1.4.1 General considerations

Proliferation is one of the most fundamental of biological processes because of its role in growth and in maintenance of tissue homeostasis (Figure 2). The cell cycle machinery is generally governed by the cyclical activation of the cyclin-dependent kinase complexes (CDKs). CDKs are a family of hetero-dimeric serine/threonine kinases that are essential for the progression of the cell cycle at every phase transition of the division process. In addition, they have distinct roles in regulating transcription. CDKs need to be complexed with their activating partners, cyclins, to exert their role on cell proliferation. CDKs, associated cyclins and CDK inhibitors act in a coordinated manner to achieve cellular homeostasis. Dysregulation of the CDKs and/or associated cyclins have been often found in tumours (Tashiro et al 2007). Cyclin D1 is a proto-oncogene that responds to extracellular mitogens and is a controller of G1 phase progression (Thomas et al 2005). The most commonly reported alteration of cyclin D1 is gene amplification (11q13). This results in the expression of a structurally normal protein, but at abnormally high levels, which leads the cell to a state of uncontrolled proliferation.

The prognostic significance of cyclin D1 overexpression/amplification has been showed in studies on laryngeal cancer (Bellacosa et al 1996), anterior tongue cancer (Bova et al 1999) and oral cancer (Miyamoto et al 2003). Ki-67 (MIB-1) is a monoclonal antibody that binds to a protein that is expressed during the G1, S and G2 phases of the cell cycle. A high percentage of immuno-labelled cancer cells for Ki-67 has been significantly associated with neck metastases and inversely correlated with the degree of cancer cell differentiation (Liu et al 2003). In order to continue cell cycling to the next phase, the prior phase must be properly completed; otherwise, safety mechanisms, also known as “cell cycle checkpoints” are activated (Paulovich et al 1997). Oncogenic transformation leads to cell cycle aberrations. When assessing tumour growth, notice should be taken such that the net growth is a result of cell gain by proliferation and cell loss by apoptosis or necrosis. Aberrations in the regulation of both cell death and cell cycle control may lead to tumour progression.

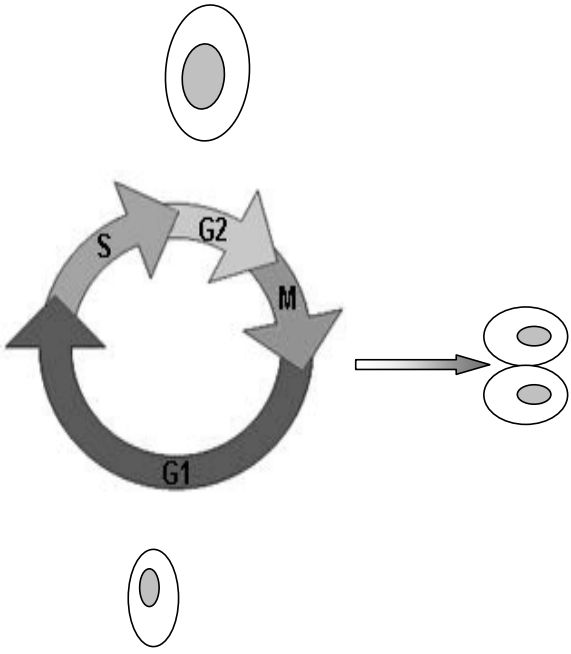

Figure 2. Synthesis of DNA (genome duplication), also known as S phase, is followed by separation into two daughter cells (M phase) in cell cycle. During the time between these phases (G_2 phase) cells can repair errors that occur during DNA duplication. The G_1 phase (time between M and S phase) represents the period of commitment to cell cycle progression.

1.4.2 Epidermal growth factor receptor

A characteristic of cancer cells is their ability to undergo extensive proliferation through overproducing growth factors and/or over-expressing receptors for growth factors. Furthermore, negative regulators of cell cycle progression can be non-functional in cancer cells, further enhancing tumour development. The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that plays a vital role in cell growth, migration, metabolism differentiation, and survival. The family of tyrosine kinases receptors also includes erbB2/HER-2, erbB3/HER-3 and erbB4/HER-4. These receptors are anchored in the cell membrane and share a similar structure that is composed of an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and an intracytoplasmic tyrosine kinase domain (Harari et al 2007). There are six known ligands that bind to EGFR, including EGF itself and transforming growth factor-α (TGF-α). Ligand binding induces a conformational change of the receptor ectodomain that allows for receptor dimerization and autophosphorylation of several tyrosine residues within the COOH-terminal tail of receptors (Burgess et al 2003). The resulting phosphotyrosines recruit the SH2 domains of multiple downstream signalling molecules and initiate an array of intracellular signalling pathways. The receptor signal is terminated by endocytic internalisation, the ligand is then degraded and the receptor recycled to the plasma membrane.

Many studies have demonstrated that EGFR is over-expressed in a number of human solid tumours including head and neck cancer (Santini et al 1991). The receptor is activated by ligand-dependent and ligand-independent mechanisms, as well as receptor over-expression (Yarden and Sliwkowski 2001). EGFR-related cell proliferation may be regulated by the tumour suppressor p53 since there is a p53-responsive site in the promoter of EGFR gene (Sheikh et al 1997). However, a p53 independent delay in cell cycle progression and a G1 arrest was demonstrated when oral cancer cells were treated with epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) (Lee et al 2007). Deletion of the extracellular receptor domain may result in constitutive, ligand-independent receptor activation (Ekstrand et al 1994). Cellular stress, such as radiation, may induce auto-activation of EGFR resulting in cell proliferation (Todd et al 1999). In addition, EGFR may escape internalization and lysosomal degradation and translocate to the nucleus, where it functions as a transcription factor of the *cyclin D* gene (Lin et al 2001). There is some evidence that cleavage of membrane-anchored ligands (e.g. TGF-α), results in autocrine/paracrine activation of EGFR, which can be prevented by matrix metalloproteinase inhibitors (O-charoenrat et al 2002). The link between EGFR and proteases was also demonstrated by Schenk et al (2003) who showed that activation of EGFR occurred through metalloproteinase-cleaved extracellular matrix particles such as laminin-5. The prognostic value of EGFR over-expression has been studied in several combination therapy trials reviewed by Lothaire et al (2006). Seven of eight trials showed a poorer outcome for patients with EGFR overexpression in multivariate analysis compared to those patients without EGFR overexpression.

1.4.3 Matrix metalloproteinases

Extracellular proteinases are required for numerous developmental and disease-related processes. Matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases collectively capable of degrading essentially all components of extracellular matrix (ECM). They share a common catalytic core with a zinc molecule in the active site. The general structure of MMPs consists of five domains as shown in Figure 3. MMPs are highly regulated and are expressed at low levels in normal tissue. They play an important role in the physiological degradation of ECM, such as tissue morphogenesis, tissue repair and angiogenesis. When an active tissue remodelling is needed, their production and activation is rapidly induced (Nagase and Woessner 1999). Regulation occurs at multiple levels, including transcription, modulation of mRNA half-life, secretion, relocalization, zymogen activation and inhibition of proteolytic activity (Sternlicht and Werb 2001). The majority of MMPs are secreted as zymogens that require extracellular activation in order to cleave their substrates. Currently, over 20 members of the human MMP gene family are known and have been classified into subgroups based on their structure and substrate specificity (Table 1). Endogenous inhibitors of MMPs are termed tissue inhibitors of matrix metalloproteinases (TIMPs, Mannello and Gazzanelli 2001), and certain non-specific proteinase inhibitors, such as alpha2-macroglobulin inhibit MMP activity (Sottrup-Jensen and Birkedal-Hansen 1989).

1.4.3.1 MMPs in tumour progression

In addition to the physiological function of MMPs described above, MMPs also have important functions in pathologic conditions characterised by excessive degradation of ECM, such as rheumatoid arthritis, osteoarthritis, periodontitis, autoimmune blistering disorders of the skin and in tumour invasion and metastasis (Nagase and Woessner 1999, Shapiro 1998, Westermarck and Kähäri 1999, Stamenkovic 2003). MMPs are abundantly expressed in various malignant tumours but several reports demonstrate that neighbouring stromal cells could be induced by the tumour cells to produce proteases (such as MMPs) that contribute to the tumour growth (MacDougall and Matrisian 1995). Moreover, the tumour microenvironment may drive

the onset of cancer. Radisky et al (2005) provide a detailed mechanistic insight into the oncogenic activity of MMP-3 when the enzyme induces production of reactive oxygen species in normal mammary epithelial cells and ultimately leads to development of squamous cell carcinomas. Ishikawa et al (2004) showed that MMP-2 status in stromal fibroblasts, not in tumour cells, was a significant prognostic factor associated with angiogenesis in non-small-cell lung cancer. Conversely, the basal MMP expression observed in cultured tumour cells can be modified by stromal constituents, such as growth factors, and cytokines. The cross-talk between different cell types in the tumour micro-environment is evident as MMPs participate in the release of matrix sequestered angiogenic and mitogenic factors that affect both tumour cells and cells in the microenvironment (Egeblad and Werb 2002).

In many tumours, infiltration of inflammatory cells is a prominent feature and these cells secrete both MMPs and cytokines, which in turn may enhance expression of MMPs in tumour and stromal cells. Proteases also participate in the regulation/dysregulation of the immune system by activating TGF-β, releasing TNF-α and degrading chemokines, cytokines and cytokine receptors, such as IL-2Rα (Sheu et al 2001). There is also evidence that receptors, e.g., c-erbB-2, TNF-α receptor, and FGFR1 (fibroblast growth factor receptor type 1), can be cleaved by matrix metalloproteinases (Molina et al 2001, Williams et al 1996, Levi et al 1996). The involvement of MMPs in degradation of receptors important for apoptosis and tumour growth, such as Fas and EGFR, is not largely investigated.

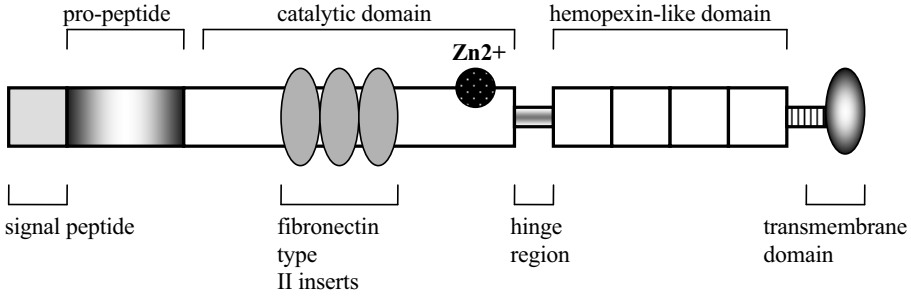

Figure 3. Prototype of MMPs (modified from Birkedal-Hansen et al, 1993). The enzyme contains a signal peptide for secretion, a pro-peptide, a catalytic domain, a hinge region and a hemopexin-like domain.

Table I. Human matrix metalloproteinases, their chromosomal localization and substrates

Enzyme	Chromosomal location	Substrates
MMP-1 Collagenase-1	11q22.2-22.3	Collagen I, II, III, VII, VIII, X, aggregan, serpins, 2M
MMP-2 Gelatinase A	16q13	Gelatin, collagen I, IV, V, VII, X, FN, tenascin, fibrillin, osteonectin, monocyte chemoattractant protein 3
MMP-3 Stromelysin-1	11q22.2-22.3	Collagen IV, V, IX, X, FN, elastin, gelatin, laminin, aggrecan, osteonectin, 1PI, myelin basic protein, OP, E-cadherin
MMP-7 Matrilysin	11q22.2-22.3	Elastin, FN, laminin, nidogen, collagen IV, tenascin, versican, 1PI, O E-cadherin, TNF
MMP-8 Collagenase-2	11q22.2-22.3	Collagen I, II, III, aggregan, serpins, 2M
MMP-9 Gelatinase B	20q12-13	Gelatin, collagen IV, V, VII, XI, XIV, elastin, fibrillin, osteonectin 2
MMP-10 Stromelysin-2	11q22.2-3	As MMP-3
MMP-11 Stromelysin-3	22q11.2	Serine proteinase inhibitors, 1PI
MMP-12 Metalloelastase	11q22.2-22.3	Collagen IV, gelatin, FN, laminin, vitronectin, elastin, fibrillin, 1-PI, myelin basic protein, apolipoprotein A
MMP-13 Collagenase-3	11q22.2-22.3	Collagen I, II, III, IV, IX, X, XIV, gelatin, FN, laminin, large tenascin aggrecan, fibrillin, osteonectin, serpins
MMP-14 MT1-MMP	14q12.2	Collagen I, II, III, gelatin, FN, laminin, vitronectin, aggrecan, perlecan, fibrillin, 1PI, 2M, fibrin
MMP-15 MT2-MMP	16q12.2	FN, laminin, aggrecan, tenascin, nidogen, perlecan
MMP-16 MT3-MMP	8q21	Collagen III, FN, gelatin, casein, cartilage proteoglycans, laminin-1, 2M
MMP-17 MT4-MMP	12q24	Fibrin, fibrinogen, TNF precursor
MMP-19	12q14	Gelatin, aggrecan, COMP, collagen IV, laminin, nidogen,
MMP-20 Enamelysin	11q22	Amelogenin, aggrecan, COMP
MMP-21 stromelysin		
MMP-23	1p36	McaPLGLDpaARNh2 (synthetic MMP substrate)
MMP-24 MT5-MMP	20q11.2	Proteoglycans
MMP-25 MT6-MMP	16p13.3	Collagen IV, gelatin, FN, fibrin
MMP-26 matrilysin		
MMP-28	17q11.2	Casein

FN, fibronectin; 2M, 2-macroglobulin; 1PI, 1-proteinase inhibitor; COMP, cartilage oligomeric matrix protein; TACE, TNF-converting enzyme; OP, osteopontin.

1.4.3.2 Importance of matrix metalloproteinases in head and neck cancer

Several studies show an increased expression and/or secretion of MMP in head and neck cancer (reviewed in Rosenthal and Matrisian 2006). High expression of MMP-2 and -9, which degrade type IV collagen, are related to tumour invasion capacity in oral carcinoma *in vivo* (Ikebe et al 1999, Kurahara et al 1999). MMP-1 was detected in fibroblastic cells of tumoral stroma and in peripheral cells of neoplastic islands in oral SCC and lymph node metastasis; meanwhile the level of MMP-1 was consistently low in normal mucosa (Sutinen et al 1998). Significantly higher levels of MT1-MMP and MMP-2 mRNA were found in HNSCC compared to control tissue (Imanishi et al 2000), and MMP-13 was only expressed in HNSCC-derived cell lines and tumour samples and not in normal skin or oral mucosa (Johansson et al 1997). Several molecules, including cytokines, may enhance MMP expression and activity. Addition of c-erbB ligands to squamous cell carcinoma cultures induced a short-term increase of MMP-1 mRNA that declined within 24 hours (O-charoenrat et al 1999). Moreover, incubation with EGF-like ligands up-regulated MMP-9 mRNA expression and gelatinase activity (O-charoenrat et al 2000).

MMPs might have prognostic significance in squamous cell carcinoma of the head and neck. Microarray studies have revealed that increased expression of MMP-1 and MMP-3 in oral SCC correlate to lymph node metastasis (Nagata et al 2003). Increased MT1-MMP and MMP-2 protein expression was correlated to metastasis and poor prognosis in SCC of the tongue (Yoshizaki et al 2001). In a study of 106 patients with advanced head and neck cancer, poor overall survival correlated with over-expression of MMP-2 protein (Ondruschka et al 2002). In addition, patients with increased secretion and activity of MMP-2 and MMP-9 had a shorter disease-free survival following treatment of oral SCC compared to patients with tumours exhibiting low MMP activity (Yorioka et al 2002). Expression of MMP-9 has also been shown to correlate with advanced T stage, lymph node involvement in a study on 54 patients with HNSCC (O-charoenrat et al 2001), and similar results were found by Dunne et al (2003). Poor survival correlated with increased MMP-9 staining in a study on 52 HNSCC patients (Riedel et al 2000). Taken together, increased active MMP-2 and MMP-9 seem to have prognostic importance in laryngeal and oral cancer (Bogusiewicz et al 2003, Yorioka et al 2002). However, recent observations have changed our understanding of how MMPs function in tumorigenesis. MMPs in general have both tumour promoting and inhibitory effects (DeClerck et al 2004), which underlines the complexity of factors involved in tumour progress.

1.5 INFLAMMATION AND CANCER

While inflammatory processes are usually evoked to destroy pathogens or initiate repair, they have also been reported to be in close association with the invading edges of aggressive neoplasias contributing to the idea that tumours are “wounds that do not heal” (Dvorak 1986). Several pro-inflammatory gene products have been identified to mediate a critical role in apoptosis suppression, proliferation, angiogenesis, invasion, and metastasis. Among these are interleukins (IL-1α, IL-1β, IL-6, IL-8, IL-18), chemokines, TNF (tumour necrosis factor) and members of its superfamily, as well as MMP-9, VEGF, COX-2 and 5-LOX (Aggarwal et al 2006).

1.5.1 The intratumoral milieu

The stromal microenvironment is complex and contains several cell types including fibroblasts, smooth muscle cells, immune and inflammatory cells, lipocytes and endothelial cells. The extracellular matrix (ECM) behaves as a reservoir of soluble and insoluble signalling molecules as well as a mechanical platform for cell attachment and movement. Tumorigenesis has been

regarded as an autonomous process in which progressive genetic derangement renders cells independent of the external context. However, there is accumulating evidence for a critical participation of tumour stroma in carcinoma development and progression (Beacham and Cukierman 2005). A complex interplay between soluble and insoluble mediators such as ECM/stroma plays a pivotal role in the control of the biological phenotype of a cell, both in normal development and function, and in pathological responses. Thus, the tumour cells and their surrounding stromal components constitute an actively communicating environment. The subsidizing role of tumour micro-environment is further questioned since there is new compelling evidence on the active role the micro-environment plays in the initial steps of epithelial tumorigenesis (Radisky et al 2005, List et al 2005). Radisky et al showed that MMP-3 enhanced the production of reactive oxygen species in normal mammary epithelial cells resulting in genomic instability. Moreover, an epithelial junctional adhesion molecule, E-cadherin, was transcriptionally down-regulated, which caused disruption of cell-cell contacts and results in a more aggressive phenotype. Moreover, there is substantial evidence for the contention that tumour-associated fibroblasts (TAFs) produce a tumour-supportive ECM, which promotes the growth, expansion and dissemination of the pre-neoplastic epithelial cell population, creating a permissive "pasture" for the emerging malignant cells (Kunz-Schughart and Knuechel 2002).

1.5.2 Cytokines as signal mediators

Cytokines are a family of peptides that are responsible for direct cell-to-cell communication. They provide a communication system between adjacent cells (paracrine effect), between cells at distant sites (endocrine effect), and self-stimulating effects (autocrine effect). One of the crucial aspects of the tumour micro-environment is the cytokine-mediated communication between tumour and stromal cells/components. Cytokines may contribute to the therapeutic benefit by stimulation of immune effector cell proliferation, augmentation of lymphocyte toxicity, and enhancement of antigen presentation of tumour-associated antigens as well as direct anti-proliferative effects (Feliciani et al 1996). The cytokine profile of cancerous tissue is commonly deranged resulting in tumour progress. Secreted pro-inflammatory chemokines and cytokines began a cycle of angiogenesis stimulation, sustained tumour growth and facilitated metastasis (Yan et al 2006). Among cytokines of central importance are interleukins-6 and -8, TNF and HGF, as described below.

1.5.2.1 Interleukin-6

Interleukin-6 (IL-6) is a multifunctional acute phase protein that is produced in response to inflammation, stress, injury and infection. It is expressed by many cell types and can co-stimulate T-cells and NK cells, and activate B-cells (Feliciani et al 1996). IL-6 is overexpressed in many types of cancer (Trikha et al 2003), and is able to promote tumour growth by up-regulating anti-apoptotic and angiogenic proteins. High levels of serum IL-6 is associated with worse survival in metastatic breast cancer (Salgado et al 2003), and in renal cell carcinoma (Costes et al 1997). Furthermore, IL-6 has been shown to have a different impact on melanoma cell proliferation based on the stage of tumour cell of development (Lu et al 1992).

The IL-6 receptor (IL-6R) consists of a signal-transducing molecule (gp130) and a specific ligand-binding protein. The latter molecule can be membrane-bound or soluble (sIL-6R), and both forms interact with IL-6 and associate with gp130 (Barille et al 2000). The soluble form of the receptor is suggested to arise via proteolytic cleavage or alternative splicing (Mullberg et al 1993, Mullberg et al 1994, Oh et al 1996, Horiuchi et al 1994). Unlike other soluble cytokine receptors, which are generally antagonists, sIL-6R is an agonist molecule, promoting IL-6 activity (Trikha et al 2003). There is some evidence that IL-6, acting through the gp130

receptor, can promote aberrant growth in HNSCC cell lines in an autocrine/paracrine loop (Sriuranpong et al 2003).

1.5.2.2 Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a soluble 17 kDa molecule that binds to tumour necrosis factor-receptor-I or -II (TNFRI and TNFRII) localised on the cell surface (MacEwan et al 2002). TNF-α is synthesised as a membrane-bound pro-peptide and is secreted upon cleavage by TNF-α converting enzyme (TACE). Additionally, the membrane-bound form of TNF-α is functional and binds to the receptor via direct cell-to-cell contact. Activated macrophages are a major source of TNF-α, although it can also be made by a variety of other cells, including fibroblasts, keratinocytes and tumour cells (Feliciani et al 1996). Evidence for a role of TNF-α in human cancer has been provided by several studies (referenced in Szlosarek and Balkwill 2003). TNF-α acts as a growth factor in certain tumour types increasing concentrations of positive cell-cycle regulators (and decreasing levels of CDK inhibitors) and components of growth-factor receptor signalling pathways such as RAS or c-MYC (Tselepis et al 2002, Gaiotti et al 2000). TNF-α promotes DNA damage and inhibits DNA repair by up-regulating nitric oxide-dependent pathways (Jaiswal et al 2000). TNF-α can induce collapse of tumour vasculature by increasing angiostatin (Weichselbaum et al 2002), but it can also promote angiogenesis (Leibovich et al 1987). TNF-α promotes further tumour remodelling by stimulating fibroblast activity, tumour-cell motility and tumour invasion via the induction of matrix metalloproteinases (reviewed in Szlosarek and Balkwill 2003).

1.5.2.3 Hepatocyte growth factor

Hepatocyte growth factor *(*HGF) was originally identified in hepatocytes but expression has been demonstrated in several tissue types including monocytes and fibroblasts, but not epithelial cells (Zarnegar and Michalopoulos 1995). The HGF receptor (C-met proto-oncogene), however, is mainly found on epithelial cells (Zarnegar and Michalopoulos 1995). HGF, secreted by fibroblasts, has a paracrine effect on epithelial cells, and stimulate re-epithelialisation in wound healing (Trusolino et al 1998). The binding to its receptor results in loss of adhesion, increased cell motility and invasiveness (Rubin et al 2001, Atabey et al 2001). HGF has been found to protect a variety of epithelial and other cancer cell types against cytotoxicity and apoptosis induced by DNA damage (Gao et al 2001, Skibinski et al 2001, Liu et al 1998). The mechanism of this action has been proposed to involve interaction between the HGF receptor and the Fas death receptor that hampers apoptosis. Wang et al (2002) showed that the HGF receptor was able to bind and sequester FasR in hepatocytes. Furthermore, mice lacking the *c-met* gene in hepatocytes were hypersensitive to Fas-induced apoptosis (Huh et al 2004) indicating an anti-apoptotic function of HGF/c-met signalling. Over-expression of HGF-receptor has been reported in oral squamous cell carcinoma, and HGF concentration in metastatic cancer tissues was significantly higher than in non-metastatic cancer tissues (Morello et al 2001, Uchida et al 2001). Interestingly, serum levels of HGF were significantly higher in oral cancer patients than in healthy volunteers (Uchida et al 2001).

1.5.2.4 Interleukin-8

Interleukin-8 (IL-8) is an 8 kDa chemokine with endothelial cell chemotactic and proliferative activity (Koch et al 1992) that promotes angiogenesis. It activates lymphocyte function-associated antigen-1 (LFA-1) on lymphocytes and lymphocyte binding to ICAM-1 on endothelium through the IL-8 receptor. Interleukin-8 is mainly secreted by monocytes and macrophages, but also fibroblasts and keratinocytes. Watanabe et al (2002) demonstrated secretion of IL-8 from oral SCC cell lines, whose production was significantly enhanced after addition of TNF-α. However, TNF-α did not exert any effects on IL-8 receptors. Expression of

IL-8 by human melanoma cells correlates with their metastatic potential (Ugurel et al 2001). Bancroft et al (2002) showed that IL-8 and vascular endothelial growth factor (VEGF) expression in HNSCC cell lines was inhibited by antagonists to EGFR. Taken together, high IL-8 expression levels render tumour cells highly tumorigenic, angiogenic and invasive (Kitadai et al 1999, Singh and Varney 2000), which makes IL-8 an attractive target for treatment of cancer (Xie et al 2001).

1.5.3 Intercellular adhesion molecule-1

Intercellular adhesion molecule-1 (ICAM-1) is an intercellular adhesion molecule that is widely distributed on many cell types of both hematopoietic and non-hematopoietic lineage. ICAM-1 is commonly expressed on vascular endothelium, lymphoid follicle germinal centres and tissue macrophages (Holtzmann et al 1988), while under normal conditions, the constitutive expression of this antigen is low. In areas of inflammation, however, cell-surface expression of ICAM-1 is dramatically increased (Rothlein et al 1988, Lisby et al 1989). LFA-1 and Mac-1, members of the leukocyte integrin family, bind to ICAM-1 (Wawryk et al 1989). This constitutes the first step of cell-to-cell interactions that is an antigen-independent adhesion between lymphocytes and antigen-presenting cells (APC), other cells or tumour targets. Expression of ICAM-1 has also been identified in human cancers, both in tissue cultures and tumour cells *in situ* (Melendez et al 2003, Esposito et al 2002). Wang et al (2006) recently showed that blocking ICAM-1 by a monoclonal antibody against this adhesion molecule growth of uveal melanoma in a SCID mouse model was inhibited. Head and neck cancer biopsies have shown positive membrane staining for ICAM-1 on tumour cells with the strongest expression in areas proximal to inflammatory cells (Lang et al 1999).

1.6 NEW TREATMENT MODALITIES

Despite aggressive cancer treatment regimens, primary or acquired resistance still constitutes a major clinical problem in medical oncology. Generally upon tumour relapse, patients usually have tumours that are more resistant to therapy than their primary tumours. The chemotherapeutic arsenal in the treatment of head and neck cancer is quite limited, which makes development of new therapeutic agents desirable.

1.6.1 Therapeutic agents affecting cell cycle control and apoptosis

Inhibition of cyclin-dependent kinase complexes (CDKs) may promote different phenotypes including cell cycle arrest, induction of differentiation, apoptosis and inhibition of transcription (Senderowicz 2003). CDK inhibitors have been tested in clinical trials (Senderowicz 2003). In order to sensitize cells to apoptosis, stimulation of pro-apoptotic or inhibition of pro-survival pathways could be accomplished. Modulation in pro-survival pathways includes inhibition of growth receptors. The involvement of growth receptors such as EGFR in epithelial tumour growth signalling has opened possibilities for new biological strategies that target EGFR and its downstream signal transduction pathways. Such therapeutic agents include ligand-linked toxins, antibodies to EGFR, tyrosine kinase-specific inhibitors, and antisense approaches to block EGFR expression. Several EGFR-specific monoclonal antibodies have been developed that bind to the extracellular domain of the receptor and have increased specificity compared with the natural ligand (TGF-β). Tumour regression has been shown after injecting HNSCC tumours with anti-EGFR or anti-Her-2-neu Pseudomonas exotoxin *in vivo* (Azemar et al 2000).

Interestingly, a potential synergism with radiation therapy and anti-EGFR chemotherapy has been reported (Harari et al 2001, Burtness et al 2005). Tyrosine kinase-specific inhibitors competitively inhibit autophosphorylation of the catalytic domain of EGFR leading to increased apoptosis (Partik et al 1999). Antisense strategy targeting the translation start site of EGFR *in vitro* inhibited the growth of HNSCC cell lines (Rubin Grandis et al 1997). Bonner et al (2006)

showed in a prospective randomised study that the addition of EGFR inhibitor cetuximab (Erbitux) during radiotherapy resulted in a significant improvement of both loco-regional tumour control and overall survival. Importantly, they did not report a higher incidence of grade 3 or higher radiation-induced side effects, such as mucositis. However, incidences of acneiform rash and infusion reactions were significantly higher among patients that received cetuximab in addition to radiotherapy. Another strategy for sensitizing tumours to apoptosis induction is to decrease the expression of anti-apoptotic proteins of the Bcl-2 family. First clinical trials with the antisense therapy agent Oblimersen (Genasense, G3139) targeted against Bcl-2 have been conducted in advanced renal cancer (Margolin et al 2007), colorectal cancer (Mita et al 2006), myeloma (Badros et al 2005), prostate cancer (Tolcher et al 2005) and lung cancer (Rudin et al 2004).

The specific targeting of death receptors to trigger apoptosis in tumour cells is an attractive cancer therapy since death receptors have a direct link to the death machinery of the cell (Ashkenazi 2002). Furthermore, apoptosis triggered by death receptors is considered to occur independent of the cellular p53 status, which is impaired in the majority of human tumours (El-Deiry 2001). Still, tumour cells might acquire treatment resistance by multiple mechanisms, which interfere with the death receptor pathway, e.g., by an increase in anti-apoptotic molecules or by a decrease in pro-apoptotic proteins. Modulation of pro-apoptotic pathways includes restoration of p53 function (Wiman et al 2006) and utilizing of TNF-α and other death receptor agonists (van Horssen et al 2006).

1.6.2 Immune therapy

The goal of immunotherapy is to enhance the natural immune response to neoplasms. Continued research in the immunobiology of solid tumours has yielded hope that the body´s immune-defense mechanisms may contribute to the available therapeutics for cancers through the use of tumour vaccines, immune response modifiers, cytokine gene therapy, and the use of specific tumour antigens (Whiteside 2006).

The complex of humoral factors and immune cells comprises two interwoven systems, innate and acquired. Immune cells scan the occurrence of any molecule that could be considered as non-self. Transformed cancer cells expressing tumour-associated proteins acquire antigenicity that is recognized as non-self by the immune system. A specific immune response is generated that results in proliferation of antigen-specific lymphocytes. Immunity is acquired when antibodies and T-cell receptors are expressed and up-regulated through the formation of lymphokines, chemokines, and cytokines. Both innate and acquired immune systems interact to initiate antigenic responses against carcinomas. The micro-environment of the developing, and especially of the advanced, tumour is, however, not supportive of immune effector cells and may favour their demise, resulting in immune suppression.

There are many obstacles in the development of effective immunotherapy in solid cancers, including head and neck cancer. The fact that a neoplasm has grown to become clinically evident implies that the tumour cells have found a way to evade the normal protective action of the immune system. There are three principles of escaping the immune surveillance: (i) escape by loss of recognition, (ii) escape by loss of susceptibility and (iii) escape by induction of immune suppression (Malmberg and Ljunggren 2006). Strategies for restoring immune competence in patients with head and neck cancer and for preventing tumour escape are necessary for more effective control of tumour progression. New immunotherapeutic approaches include cytokine- and dendritic cell-based vaccines. These are aimed to restore tumour-specific responses and to protect immune cells from tumour-induced apoptosis (Whiteside 2005).

1.6.3 Cytotoxic and immuno-modulatory agents

Initially, anti-tumour chemotherapies were thought to produce solely anti-proliferative or cytotoxic effects on the dynamically dividing cells, among which tumour cells were the desired targets. However, more recent studies have shown that chemotherapeutic agents are capable of sensitizing the immune response in a dose-dependent manner (Maguire and Ettore 1967, Schwartz and Grindey 1973). In cancer patients, most of the anti-tumour chemotherapeutics are applied at maximum tolerated doses (MTD), which can cause immuno-suppression. Instead, there is increasing evidence that low to moderate doses of cisplatin induce immuno-augmentation (Spreafico and Vecchi 1985, Kleinerman and Zwelling 1984, Schlaefli et al 1983). Cisplatin is also known to make the tumour cells more susceptible to immune cell attack (Noguchi et al 1997). One of the mechanisms proposed is sensitization of tumour cells to apoptotic signal transduced through the Fas-FasL-mediated pathway (Michaeu et al 1997, Uslu et al 1996).

Cisplatin is an effective chemotherapeutic agent widely used in the treatment of many varieties of malignancies (Lebwohl and Canetta 1998). Cisplatin is a potent radiosensitizer and the drug most commonly used for chemoradiotherapy in head and neck cancer (Seiwert et al 2007). The cytotoxicity of cisplatin is thought to be a result of formation of inter- and intra-strand DNA cross-links (Eastman et al 1990). However, more recent studies show that cisplatin induces caspase-3 activation and apoptosis by activation of ASK1 and the stress-induced protein kinase (SAPK) pathway (Chen et al 1999). Furthermore, cisplatin was reported to stimulate apoptosis by inhibiting XIAP, a direct inhibitor of caspase-3, -6 and -7 (Matsumiya et al 2001). Yang et al (2006) showed that cisplatin binding to nuclear DNA is not necessary for induction of apoptosis in squamous cell carcinoma of the head and neck, which can instead result from the direct action of cisplatin on mitochondria. They showed that cisplatin binds preferentially to mitochondrial membrane proteins, particularly the voltage-dependent anion channel. A release of cytochrome c was observed after cisplatin treatment.

1.6.3.1 Interferon

Interferons (IFN) were isolated in 1970 and named IFNs because they interfered with viral infection. Interferon-α (IFN-α) is a family of molecules comprising at least two types, and they have profound and diverse effects on gene expression. IFN-α may be very important in linking innate-immune response with sustained adaptive-immune response (Biron et al 1998). The innate-immune response usually consists of the cell-mediated response of NK-cells to non-self (e.g. neoplastic) or foreign (e.g. viral) antigen. Although important for initial defence of the host, the innate-immune response must transition to the more efficient and specific adaptive-immune response to clear the non-self or foreign antigen effectively. In this process, IFN-α is considered critical in the regulation of the immune response transition by direct or indirect regulation of activity of cytokines and chemokines (Taylor and Grossberg 1998, Biron et al. 2001), and by regulation of cytokine- and chemokine-receptor expression at the cell surface (Rogge et al 1997). Other actions ascribed to IFN-α is an anti-angiogenic effect, activation of B- and T-cells and antigen-presenting cells, and increased expression of class I MHC antigens (Harris and Gill 1986), tumour antigens and adhesion molecules, The efficacy of IFN-α has been well established for the treatment of patient having advanced malignant melanoma and renal cell carcinoma (Bukowski et al 2002).

1.6.3.2 Retinoids

Retinoids are natural and synthetic derivates of vitamin A. The natural retinoids exert a hormone-like activity and function in several processes including vision, reproduction, metabolism, growth, differentiation, hematopoiesis, immunological processes, bone development, and pattern formation during embryogenesis (Sporn et al 1994).

Epidemiological studies have addressed the relationship between retinoids and cancer incidence. Serum levels of β-carotene (pro-vitamin A) and vitamin A (retinol) were significantly higher in control subjects than in oral cancer patients (Kune et al 1993, de Vries and Snow 1990). There is considerable evidence that retinoids have potent growth inhibiting effects on cancer *in vitro* and *in vivo* (Gudas et al 1994). They have been used in treatment of oral leukoplakia, a premalignant mucosal lesion that frequently develops into invasive oral SCC (Hong et al 1986). Despite that significant response to high-dose therapy was observed, a substantial toxicity expressed as mucocutaneous reactions, liver toxicity and myalgia was found. Moreover, a high rate of relapse after discontinuation of the treatment hampered the clinical use of this drug. Head and neck cancer patients have a 2-4% risk of developing second primary tumours every year. Patients that were treated with an adjuvant 12-month 13-cis retinoic acid (13-cis RA) therapy after curative surgery and/or radiation therapy developed significantly fewer second primary tumours after 32 months of follow-up (Hong et al 1990). Upon re-analysis after a median 4.5 years, 13-cis RA-patients continued to have significantly fewer total second primaries, suggesting that the protective effect lasted for several years after completion of the therapy (Benner et al 1994). The effect may be dose-dependent since a recently reported randomized phase III trial of low-dose 13-cis retinoic acid for prevention of second primary tumours in stage I and II head and neck cancer patients did not show any reduction of the rate of second primary tumours or death (Khuri et al 2006). Interestingly, Hoffmann et al (2007) showed that 13-cis retinoic acid inhibited angiogenesis and tumour growth in thyroid cancer cells. In addition, retinoids may be effective in cancer therapy by blocking MMP synthesis and decreasing tumour invasiveness (Schoenermark et al 1999).

1.6.4 MMP-inhibitors

In preclinical studies, broad spectrum synthetic MMP inhibitors have shown significant anti-tumour activity. The large-scale randomized phase III trials have, however, failed to confirm the effects, reviewed in Coussens et al (2002). Although the development of specific MMP inhibitors may result in more effective anticancer compounds, the MMP system is extremely complex and it is not yet clear which subtypes that should be targeted to achieve therapeutic anti-tumour effect (Hojilla et al 2003).

1.6.5 Other treatment agents

Numerous biological and small-molecule compounds capable of modulating TNF function are either available commercially, or are under clinical evaluation. Several of the TNF antagonists are approved for other indications than cancer and are under investigation for supportive care or treatment of various malignancies (reviewed in Yan et al 2006). Furthermore, studies on anti-IL-6 therapies have been performed on patients with multiple myeloma, renal cell carcinoma and B-lympho-proliferative disorders (reviewed in Trikha et al 2003). The anti-IL-6 mAb therapy decreased C-reactive protein levels in all patients, the antibodies were well tolerated, and since the incidence of cancer-related anorexia and cachexia was decreased the antibodies may be useful in palliative treatment of cancer patients. In pre-clinical models human anti-IL-8 antibody inhibited angiogenesis, tumour growth and metastasis of human melanoma (Huang et al 2002), but the therapeutic value of blocking IL-8 has yet to be assessed in cancer patients.

2 AIMS OF THE STUDY

The general comprehensive aim of this thesis was to study different aspects of tumour progress including apoptosis, cell proliferation and inflammation in head and neck cancer.

The specific aims of the study were as follows:

Paper 1:

To study and evaluate the prognostic values of three parameters related to apoptosis and proliferation in a group of malignant salivary gland tumours (acinic cell cancer). Expression of Bcl-2 expression, proliferation and apoptosis frequency were assessed.

Paper 2:

To investigate and evaluate the prognostic values of three parameters related to apoptosis (Bcl-2, MT, Fas) in tongue cancer. A secondary aim was to assess cell proliferation and apoptosis frequency in the same tumours.

Paper 3:

To investigate the effect of low-dose chemotherapeutic agents (cisplatin, interferon-alpha, 13-cisRA) on expression of receptors Fas, EGFR and ICAM-1 in two oral squamous cell carcinoma cell lines.

To investigate how effects induced by low-dose cisplatin influences Fas-mediated apoptosis.

Paper 4:

To study secretion of matrix metalloproteinases (MMP-1, MMP-2 and MMP-9) from oral cancer cell lines and secondly, to study if different cytokines (IL-6, sIL-6R, TNF-alpha, HGF, IL-8 or their combinations) stimulate MMP secretion in these cell lines.

3 MATERIAL AND METHODS

Study population (paper 1)

32 cases with acinic cell cancer were studied, 16 from Germany and 16 cases from University Hospital in Linköping. All patients were followed for a minimum of 5 years following treatment.

Study population (paper 2)

24 consequent patients with tongue cancer (T1-T2N0M0) treated at the University Hospital in Linköping were studied. None of the patients had been exposed to any prior anti-tumour treatment , and they were followed for a minimum of 3 years.

Cell proliferation assessment (paper 1 and 2)

In the present papers, Ki-67 was detected by immunohistochemistry. First, endogenous peroxidase activity was inhibited by hydrogen peroxidase, and the sections were immersed in boiling citrate buffer in a microwave oven two times (5 minutes each). After washing, the sections were subsequently incubated with 1:100 dilution of monoclonal antibodies to Ki-67 (MIB-1, Immunotech, France) overnight at +4°C; a 1:200 dilution of biotinylated rabbit antiserum to mouse immunoglobulins for 30 min at room temperature; and a 1:100 dilution of the streptavidin-biotinylated peroxidase complex for 30 min. Peroxidase activity was developed in diaminobenzidine chromogen substrate. The MIB-1 immunoreactivity in tumour cells was evaluated either as less than 10 percent positive (=negative) or as more than 10 percent positive (=positive). Negative control sections, where the specific monoclonal antibody was replaced with the immunoglobulin fraction of non-immune mouse sera, remained unstained.

When studying cell proliferation, markers of proliferation can be classified into three main categories: growth fraction markers, markers of specific phases in cell cycle and cell cycle time markers (Pich et al 2004). The oldest way of assessing proliferation is counting mitotic cells on a light microscopy. Cells in the M phase can be identified but cells in the largest part of the cell cycle (G_1, S and G_2) cannot be morphologically recognized. The Ki-67 antigen, which is coded by a gene on chromosome 10, is expressed in the G_1, S and G_2 phases in cycling cells (Gerdes et al 1992, Guillaud et al 1989). Immunohistochemical staining of Ki-67 by MIB-1 antibody is regarded as a reliable marker of proliferating cells. There are additional proliferation associated antigens that can be visualized by immunohistochemistry, proliferating cell nuclear antigen (PCNA) and DNA topoisomerase II. Other methods to assess cell proliferation include incorporation techniques (incorporation of labelled nucleotide analogues *in vivo* or *in vitro*), DNA cytometry (percentage of cells in S phase), and silver staining of nucleolar organiser regions (AgNORs). Growth is the overall increase in cell number, so is the net result of cell gain by proliferative activity and cell loss by apoptosis or necrosis. It is recommended to assess both parts that influence growth.

Immunohistochemistry in tissue sections (Bcl-2, Fas, MT), paper 1 and 2

4-5 µm thick sections were cut from representative blocks of the original surgical specimen and deparaffinized. Next, endogenous peroxidase activity was inhibited by hydrogen peroxidase, and the sections were immersed in boiling citrate buffer in a microwave oven two times (5 minutes each). After washing, the sections were subsequently incubated with 1:20 dilution of normal rabbit serum, followed by either 1:50 dilution of monoclonal antibodies to Bcl-2; 1:25 for anti-Fas, or 1:25 for anti-MT overnight at +4°C. Sections were incubated with a 1:200 dilution of biotinylated rabbit antiserum to mouse immunoglobulins for 30 min at room temperature followed by a 1:100 dilution of the streptavidin-biotinylated peroxidase complex for 30 min, also at room temperature. Peroxidase activity was developed in diaminobenzidine

(DAB) chromogen substrate. For all antigens, tumour positivity was graded as 3+ if more than 75% of tumour cells were stained, 2+ if 25-75% of cells were stained, 1+ if <25 % of cells were stained, unless all were negative. A formalin-fixed, paraffin-embedded follicular lymphoma carrying the t(14;18) chromosomal translocation was applied as a positive Bcl-2 control. Sections of cultured human T-Jurkat cells were used as a positive control for Fas, breast tissue (containing myoepitelial cells) and a normal and malignant parotid gland were used as a positive control to MT. Negative control sections, where the specific monoclonal antibody was replaced with the immunoglobulin fraction of non-immune mouse sera, remained unstained.

Apoptosis detection (TUNEL), paper 1 and 2

After deparaffinization, sections for the TUNEL reaction were digested by 20 µg/ml proteinase K for 15 minutes. After four washes in distilled water and quenching in 2% H_2O_2, the Apoptag kit was applied according to manufacturer´s instructions. Briefly, the TUNEL method is a tailing reaction where terminal deoxynucleotidyl transferase (TdT) catalyses a template-independent addition of deoxyribonucleotide triphosphate to the 3´-OH ends of single- or double (blunt ends) –stranded DNA. The incorporated nucleotides form a random heteropolymer of digoxigenin-11-dUTP and dATP, in a ratio that has been optimized for anti-digoxigenin antibody binding. The anti-digoxigenin antibody fragment carries a conjugated reporter enzyme (peroxidase) to the reaction site. TUNEL positivity was evaluated as 1+ when less than 5 percent of tumour cells showed distinctive nuclear staining, with an intensity equalling or almost equalling that of control, 2+ if more than 5 percent but less than 25 percent of tumour cells were positive, and 3+ when more than 25 percent of tumour cells were positive. Tumours with no positive cells, or the occasional positive cell only, were recorded as negative. TdT was replaced with water in negative controls. For positive controls, DNase I was added (20 min, 37°C) after quenching in H_2O_2, thus producing DNA breaks in virtually all cells (Gavrieli et al 1992).

The TUNEL reaction is widely accepted and sensitive indicator of apoptosis at the single cell level. Its full specificity remains to be clarified, as TUNEL is a tailing reaction and theoretically could incorporate dUTP to any DNA nick break and not only those occurring during apoptosis. Combination with assessing apoptotic morphology, e.g. by visualizing nuclear condensation by DAPI staining, is recommended. The appearance on the surface of apoptotic cells of phosphatidylserine is exploited using the Annexin V assay. This is particularly effective when analysing single cell populations of tumour cells by flow cytometry (e.g., haematological malignancies).

Cell lines and culture conditions (papers 3-4)

Two recently established squamous cell carcinoma cell lines (UT-SCC-20A, UT-SCC-24A) were studied in paper 3 and 4 (Lansdorf et al, 1999), Figure 4. UT-SCC-20A has one missense mutation in exon 7 (codon 248) of the *p53* gene (Pekkola-Heino et al, 1996). UT-SCC-24A has one normal *p53* allele and one allele with splice mutation in exon 7 (Hauser et al, 2002). The cell lines were established from primary squamous cell carcinomas of the mobile tongue (UT-SCC-24A) and the floor of the mouth (UT-SCC-20A), and passages 10-20 were used for experiments in the present studies. The cells were cultured in Dulbecco´s modification of Eagle´s medium (DMEM) supplemented with 2 mM L-glutamine, non-essential amino acids, antibiotics (streptomycin 50 µg/ml, penicillin 100 IU/ml) and 10% foetal calf serum (FCS), all from Gibco, Life Technologies. All cells were incubated at 37°C in a humidified air atmosphere with 5% CO_2.

Cell detachment procedure (paper 3)

The cells were harvested at sub-confluent growth in flasks, and trypsinization or mechanical cell detachment with a cell scraper was performed in parallel flasks. A solution with 0.01% trypsin (Sigma-Aldrich, Gillingham, UK) and 0.02% K-EDTA was used for enzymatic detachment. Both trypsinized and scraped cells were prepared on cytospin glasses (7 minutes at 8.5 *g*), or, alternatively, the cells were cultured on glass chamber slides for 72 hours then directly dried and stored at –70°C until staining.

cell line	primary tumour location	specimen origin	TNM
UT-SCC-20A	base of mouth	Primary tumour	T1N0M0
UT-SCC-24A	tongue	Primary tumour	T2N0M0

Figure 4. Characteristics of the studied cell lines.

Drug modulations (paper 3)

After trypsinization the cells were seeded onto glass chamber slides, UT-SCC-20A at 40 000 cells/ml and UT-SCC-24A at 50 000 cells/ml. After 24 hours, the culture medium was changed and CDDP (0.5 µg/ml), INF-α (1000 U/ml), and 13-cisRA (0.003 µg/ml) were added. The cells were cultured with or without drugs for 48 hours and then the slides were gently washed with PBS solution, dried, and stored at –70°C.

The toxicity of cisplatin was previously studied in several cell types and the cell death curves commonly show a steep increase of cell death/apoptosis at concentrations higher than 1 µg/ml (Willliams et al 1997, Song et al 2003, Kinoshita et al 2000). Earlier studies with cisplatin treatment at concentration <0.5 µg/ml showed less than 10% cytotoxicity in HNSCC cells (Matsuoka et al 1995) whereas treatment of HNSCC cells by higher cisplatin concentration (1-5 µg/ml) showed 20-50% cytotoxicity (Matsuoka et al 1995, Kuwahara et al 2000). The higher concentrations in cell cultures are equivalent with clinical treatment concentrations. The present purpose was to study modulating, not toxicity-derived effects on receptors. Different drug concentrations were tested on cells and the concentrations of minimal effect on observed cell growth were chosen.

Immunohistochemistry on glass chamber slides (Fas, ICAM-1, EGFR), paper 3

The cell preparations on chamber slides were fixed with phosphate-buffered 4% paraformaldehyde, pH 7.4, for 5 minutes, and then blocked with 10% human AB serum for 15 minutes. The cells were incubated with primary mouse anti-human antibodies Fas 50 µg/ml (clone DX2, Dako Corp, Carpinteria, CA, USA), ICAM-1 8 µg/ml (clone 6.5B5, Dako, Glostrup, Denmark), EGFR 1 µg/ml (Labvision, Fremont, CA, USA), and IgG 50 µg/ml (IgG_1, Dako, Glostrup, Denmark). The antibody dilutions used differed depending on the purpose of the staining; the antibodies used in staining after drug modulations (paper 3) were deliberately more dilute in order to make it easier to observe any receptor up-regulation (Fas 25 µg/ml, ICAM-1 0.125 µg/ml, EGFR 0.125 µg/ml, and IgG 25 µg/ml). All primary antibodies were incubated for 30 minutes except for anti-EGFR mAb and the negative control, which were incubated for 60 minutes. After incubation with the primary antibodies, the slides were washed in BSS and Tris buffered saline (TBS) and incubated with Envision+, Peroxidase, Mouse (Dako Corp, Carpinteria, CA, USA) for 30 minutes. The slides were then washed in TBS and

incubated with AEC+ substrate-chromogen (Dako Corp, Carpinteria, CA, USA) for 20 minutes. The washing was repeated after AEC+ incubation, then the slides were counterstained in Mayer's hematoxylin for one minute and mounted in Glycergel (Dakopatts, Sweden).

There are a variety of immunohistochemical staining methods to apply. Depending on the studied sample (deparaffinized sections, frozen sections, cell samples, smears) and the studied epitope (membrane bound, intracellular, nuclear) different methods may be chosen. Staining results for a protein can depend on which epitope is chosen as a target of the primary antibody. Two methods were used in the present investigations since there were differences between samples (deparaffinized sections and cell samples on glass slides) and the time interval between analyses. The cells that were detached by trypsin prior to staining showed a stronger staining intensity than attached cells. Trypsin may promote unmasking of epitopes, which can explain the different intensity. Moreover, microwave treatment of tissue sections may intensify staining reaction, and the concentration and incubation time of primary antibodies plays an important role in staining results. Since there is plenty of variation in immunohistochemical techniques staining results of separate investigations should be compared cautiously.

Detection of soluble and cell-bound Fas (paper 3)

The cells were seeded onto 6-well plates (NUNC, Life Technologies); UT-SCC-20A at 9×10^4 cells/ml, and UT-SCC-24A at 11×10^4 cells/ml. After 24 hours of plating, the culture medium was changed in all dishes and the different modulating agents (CDDP 0.5 µg/ml, INF-α 1000 U/ml, and 13-cisRA 0.003 µg/ml) were added. After 48 hours, the cells were gently harvested using a cell scraper. The cell suspension consisting of cells and culture medium was collected and centrifuged at 420 x *g* for 5 minutes. The culture medium was collected separately and the cells were washed once in PBS. All samples were stored at –70°C until receptor quantification. The amount of Fas receptor in both membrane-bound and soluble form was determined using Fas/APO-1 ELISA (Oncogene, Calbiochem). All assays were conducted according to the suppliers' recommendations. The optical density of all samples was determined using a microplate reader (Versa max, Molecular Devices Corp, Sunnyvale, California). Protein determination of samples was performed using a bicinchoninic acid protein (BCA) assay kit (Sigma, Missouri, USA), following the detection protocols recommended by the manufacturers.

Apoptosis induction (CH11) and detection (DAPI and caspase-3), paper 3

The cells were seeded onto 6-well plates (Costar) at a density of 12,000/cm^2 (UT-SCC-20A) and 15,000/cm^2 (UT-SCC-24A). After 24 hours, fresh medium was added either with or without cisplatin (0.5 µg/ml), and after another 40 hours an apoptosis-inducing Fas antibody (clone CH11, Upstate, Lake Placid, NY, USA; 50 ng/ml) was added for 8 hours. Cells were then trypsinized and together with detached cells collected in the culture medium and centrifuged (420 x *g* for 5 minutes). The cell pellet was re-suspended in 100 µl phosphate-buffered saline (PBS) and subjected to cytospin centrifugation at 8.5 x *g* for 5 minutes. The cells deposited on slides were fixed in 4% neutral buffered formalin for 20 minutes, rinsed in water, and mounted in Vectashield® mounting medium supplemented with the fluorescent DAPI (1.5 µg/ml; Vector Laboratories, Burlingame, CA, USA), which stains DNA.

Nuclear morphology was examined in a Nikon microphot SA fluorescence microscope (Nikon, Tokyo, Japan) using UV exciting light and a blue barrier filter. Samples were blinded and the number of cells with nuclear apoptotic morphology was determined. When an apoptosis-inducing Fas antibody was added to cell cultures with cycloheximide (1 µg/ml) for the last 8 hours, apoptotic nuclei were observed. Thereafter, caspase-3 activity was measured in the cell samples. Caspase-3 activity was measured using the fluorescent substrate Ac-DEVD-7-amino-4-methyl coumarin (Ac-DEVD-AMC; Becton-Dickinson, Mountain View, CA, USA), which is cleaved by caspases with caspase-3-like activity. Measurements were performed according to

the manufacturer's instructions. After incubation for 1 hour at 37°C, the amount of released AMC was analyzed at λ_{ex} 380 nm and λ_{em} 435 in an RF-1501 Spectrofluorophotometer (Shimadzu, Kyoto, Japan). The amount of released AMC was calculated using a standard curve, and correlated to total protein determined with the method described by Lowry et al (20). Caspase-3 activity was expressed in nmol AMC/mg protein/h.

Cycloheximide was applied to an apoptosis induction assay and an increased apoptotic activity was initially observed after addition of this protein synthesis inhibitor. Mitsiades et al (2000) previously showed that Fas-mediated apoptosis did occur in thyroid carcinoma cell lines only in the presence of cycloheximide, suggesting the presence of an inhibitory protein. One logical candidate for such an inhibitor is the FLICE-like inhibitory protein (FLIP) that associates with caspase-8 and caspase-10 at the death-inducing signalling complex (Scaffidi et al 1999). The *flip* gene encodes two principal proteins, a more abundant longer isoform FLIP-L and a less prevalent shorter protein FLIP-S. FLIP-S is exclusively a caspase inhibitor but the longer form FLIP-L has a dual function as either a caspase inhibitor or activator (Chang et al 2002). Mitsiades et al (2000) showed that the agonistic anti-Fas antibody CH-11 induced FLIP-L association with Fas in thyroid cancers.

Cell culture protocol assessing spontaneous MMP secretion (paper 4)

The cells were trypsinized and seeded onto 6-well plates at a density of 13,000 (UT-SCC-20A) and 16,000 cells/cm2 (UT-SCC-24A). They were then allowed to attach for 24 hours in all experiments. Thereafter, three different experimental models were used: 1. The cells were cultured for another 24 hours in serum-free medium; 2. The cells were cultured for 24 hours in serum-containing medium followed by another 24 hours in serum-free medium; 3. The cells were cultured in serum-containing medium for 96 hours.

Cytokine modulations (paper 4)

After plating for 24 hours, serum-containing medium was changed, and IL-6 (50, 100, 200 ng/ml), sIL-6R (250, 500, 1000 ng/ml), TNF-alpha (1, 10, 100 ng/ml), HGF (50, 100, 200 ng/ml) and IL-8 (10, 100, 500 ng/ml, all cytokines were from R&D Systems, Abingdon, UK) were added. The conditioned medium was collected 96 hours later.

Detection of MMP-1, -2 and -9 (paper 4)

The conditioned media from modulated and non-modulated wells with an equal cell number were collected, centrifuged at 420 x g for 5 min to remove cellular debris and frozen at -70˚C until further processing. The conditioned media were analysed for total human MMP-1, MMP-2 and MMP-9 by MMP-1 ELISA (Amersham, Pharmacia, Biotech), MMP-2 ELISA (R&D Systems, Abingdon, UK) and MMP-9 ELISA (R&D Systems, Abingdon, UK). Detection protocols recommended by the manufacturers were followed. The optical density of all samples was determined using a microplate reader (Versa max, Molecular Devices Corp, Sunnyvale, California).

Gelatin zymography (MMP-2 and -9), paper 4

The cell cultures used in experiments for determination of MMP protein in supernatants after 120 hours in FCS medium with cytokines (as described above) were continued to determine proteolytic activity. After the supernatants were collected at 120 hours, the cells were washed with serum-free medium, and fresh serum-free medium without cytokines was added. The cells were then cultured an additional 24 hours. The supernatants were collected and frozen at -70°C until used for gelatin zymography. Conditioned medium was concentrated approximately ten times using Vivapore concentrator (Vivascience AG, Hannover, Germany) prior to gel electrophoresis. The samples were mixed with SDS sample buffer without heating or further

reduction and applied to 10% polyacrylamide gels copolymerized with gelatin (BioRad Laboratories, Hercules, California). After electrophoresis, gels were washed for 30 minutes at room temperature in renaturation buffer containing 2.5% Triton-X 100. Gels were then washed with development buffer (50 mM Tris-Cl , pH 7.5, 5 mM $CaCl_2$ and 0.02% Brij-35) and further incubated in development buffer for 18 hours at 37˚C on a shaker. Thereafter, the gels were stained with Coomassie Blue R-250 (0.5%) in 40% methanol / 10% acetic acid for 1 hour, followed by de-staining in 40% methanol / 10% acetic acid. Enzyme activity was visualised as colourless bands on a blue background.

The activity of metalloproteinase–2 and -9 (gelatinases) was assessed by gelatine zymography which is a semi-quantitative method. An enhanced enzyme activity was not detected in the present investigation until there was a considerable increase of MMP concentrations verified by ELISA technique. A more sensitive detection method, e.g. fluorescent assay, may be preferably applied.

4 RESULTS

Paper 1

Cell proliferation (Ki-67), apoptosis (DNA fragmentation) and expression of Bcl-2 were studied in 32 acinic cell cancers. The clinical staging, histological sub-classification and biological behaviour (patient outcome) were correlated with the results. Patients with stage I tumours had significantly better survival compared to patients with higher stage tumours. Bcl-2 immunostaining was performed in all cases but one. In 21 tumours (of 31), more than 75% of cells were positive. In four cases, Bcl-2 positivity was found in 25-75% of cells and two cases had less than 25% Bcl-2-positive cells. Four tumours were devoid of Bcl-2 immunoreactivity. No significant correlation was observed between Bcl-2 expression, tumour staging, or morphological subtype. Four of 16 tumours stained for the proliferation marker MIB-1 were found to be positive (more than 10% positive tumour cells). Patients with MIB-1-negative tumours had significantly better survival than patients with MIB-1-positive tumours. The frequency of spontaneous apoptosis was studied by TUNEL in 27 cases of acinic cell cancer, which was most pronounced in stage I compared with stages II, III and IV, probably indicative of greater apoptosis. Statistical analysis of correlation between different stages and TUNEL positivity failed to reach significance.

Paper 2

Metallothionein, Fas, and Bcl-2 expression were assessed in 24 cases of T1N0M0 and T2N0M0 tongue cancer that clinically represent a relatively homogenous group of tumours. Moreover, TUNEL positivity indicating spontaneous apoptosis was studied in these tumours. Metallothionein expression in the normal oropharyngeal epithelium (controls) was strictly localised to basal and parabasal cell layers. All tumour cases that were considered positive were graded as either 2+ or 3+ (> 25% positive cells) for all studied antibodies. A positive MT staining was found in 22 of 24 tongue cancer specimens and the staining was generally observed in the peripheral cells of tumour nodules mimicking the staining pattern of normal epithelium.

There was some evidence of early invasion in the MT positive tumour cells from basal areas of the epithelium. MIB-1 positivity (proliferation activity) was localized to same areas as MT in tumours, but TUNEL-positive cells were more prevalent in the central rather than peripheral areas. Five tumour specimens showed positive staining for Bcl-2, which was uniformly distributed throughout tumour nodules. All tumours were positive for Fas and the distribution pattern was similar to that of Bcl-2. MT, Fas and Bcl-2 staining were not significantly correlated with survival in the studied group of tongue cancer patients.

Paper 3

The investigation of two oral cancer cell lines was preceded by optimizing the cell preparations before immunohistochemical staining, and thereafter the cells were exposed to chemotherapeutic drugs. Cisplatin enhanced the membrane and cytoplasmic staining of Fas in both cell lines as well as the cytoplasmic staining of ICAM-1 in UT-SCC-24A. Interferon-α enhanced the staining intensity for EGFR and ICAM-1 in the cytoplasm of UT-SCC-24A cells, whereas no effect was found on Fas expression. 13-cis retinoic acid enhanced cytoplasmic and membrane staining of ICAM-1 in UT-SCC-20A and a similar effect was observed on Fas and EGFR staining in UT-SCC-24A.

Further investigation of the death receptor Fas was of particular interest in the present study. All three modulating drugs increased the amount of soluble Fas in conditioned medium of UT-SCC-20A whereas the amount of cell-bound receptor was not affected. Drug modulations had a

different effect on UT-SCC-24A since no increase of either soluble or cell-bound receptor was observed. The effect of low-dose cisplatin on the function of Fas receptor was then investigated. A low spontaneous caspase-3 activity indicating apoptosis was found in both cell lines and the addition of cycloheximide, a protein synthesis inhibitor, had a slight up-regulating effect on spontaneous apoptosis activity.

Incubation of cells with low-dose cisplatin did not increase caspase-3 activity and the agonistic Fas-antibody (CH11) had no particular effect on apoptosis until cycloheximide was added. A significant increase in caspase-3 activity was observed in both cell lines when both the apoptosis-inducing antibody CH11 and cycloheximide were added to the cell cultures. Cisplatin further increased the apoptotic activity in UT-SCC-24A.

Paper 4

The spontaneous secretion of MMP-1, MMP-2 and MMP-9 was examined in two oral cancer cell lines (UT-SCC-24A and UT-SCC-20A). The three enzymes were found to be secreted by both cell lines, however, the enzymatic secretion profile varied. The enzyme secretion induced by five cytokines was then investigated and effects were mostly observed for MMP-1 and MMP-9 secretion.

IL-6 had a moderate stimulatory effect on MMP-1 secretion in both cell lines, whereas its soluble receptor had no effect. When these cytokines were added together, a dose-dependent, synergistic stimulatory effect was observed. MMP-1 secretion was up-regulated after stimulation with HGF, especially in UT-SCC-24A. Furthermore, a synergistic effect was observed in both cell lines when HGF and IL-6 were added together. TNF-α stimulated MMP-1 secretion particularly in UT-SCC-24A cells and IL-8 showed a similar, though more moderate, effect in the same cell line. An increase in secreted MMP-9 in UT-SCC-24A was observed when stimulated with HGF or IL-6 combined with its soluble receptor. TNF-α stimulated both cell lines to increase MMP-9 secretion and a more than 10-fold stimulatory effect was observed in UT-SCC-24A. A tendency for increased MMP-2 secretion was observed in UT-SCC-24A when cells were stimulated with TNF-α, or a combination of HGF and IL-6.

5 DISCUSSION

Paper 1

Clinical staging and histopathological grading have hitherto been the most reliable prognostic indicators for acinic cell cancer and for salivary gland tumours in general. Skalova et al. (1994) correlated expression of Ki-67 (MIB-1) with prognosis in acinic cell carcinoma and the present investigation confirmed these findings in all tumours where staining was possible. Luukkaa et al. (2006) studied 212 patients with salivary gland malignancies and showed that a high volume corrected index of Ki-67 staining (Ki-67/mm^2 of tumour tissue) was associated with poor patient outcome. Similar results with correlation to prognosis were shown by Lim et al. (2003), but results have also been published that do not show Ki-67 as an independent risk factor (Okabe 2001). Generally, when immunohistochemical staining is applied and the positive staining is assessed, both scales (e.g., <25%, 25-50%, 50-75%, and >75% of cells) and cut-off points (e.g., >10% of cells) can be used to present the results and thereby a variety of presentations can be seen in the literature. Therefore, some difficulties may be encountered in interpreting and comparing results. In the present investigation, the same cut-off point for Ki-67 (MIB-1) positivity was applied as in the earlier study of Skalova et al. (1994).

Most tumours in the present study had a high level of Bcl-2 protein, and only a few totally lacked immunoreactivity. High levels of Bcl-2 were detected in all tumours from patients that died of their cancer. Yin et al. (2000) studied 71 cases with mucoepidermoid carcinoma of the oral minor salivary glands and found that patients with high Bcl-2 expression had a higher survival rate than the Bcl-2-negative group. With the contemporary knowledge of the Bcl-2 protein family, both apoptosis inducers (e.g., Bax, Bad, Bid, Bcl-X_s) and blockers (e.g., Bcl-2, Bcl-X_L) should be analysed in order to estimate whether a pro- or anti-apoptotic protein ratio predominates. The Bcl-2 family plays an important role in mitochondrial amplification of apoptosis signalling, but the amplification can be by-passed in other apoptotic pathways. It seems that the overall status of cell death machinery, rather than just the expression levels of the individual proteins, might explain how tumorigenesis is affected. Leivo et al. (2005) carried out a cDNA array analysis of gene expression in well-characterized salivary gland tumours and showed that only a few genes were deregulated in acinic cell carcinomas.

An inverse relationship between Bcl-2 and TUNEL positivity was expected, but was not seen. As discussed above, a wider perspective of apoptosis regulation and the simultaneous investigation of more factors may increase the validity of individual analyses. Eight of 12 patients with no evidence of disease in the follow-up expressed TUNEL in more than 10 per cent of cancer cell nuclei, while three patients who died lacked TUNEL positivity. Although these data failed to reach statistical significance, they may indicate that there was a tendency for a positive correlation between TUNEL positivity and better survival. These results are supported by Yin et al. (2000). Patients with mucoepidermoid carcinoma of minor salivary glands that had strong TUNEL positivity showed a higher survival rate than patients with weak or no TUNEL staining (Yin 2000). A different result, however, was recently published by Ben-Izhak et al. (2007) showing that positive TUNEL staining correlated with metastasis, extracapsular spreading, high tumour stage and worse prognosis in malignant salivary gland tumours, and if an extensive Ki-67 staining also occurred, a reduction of 5-year survival rate was observed. Moreover, Tsuchiya et al. (2001) showed that the apoptosis index before treatment had no prognostic value for oropharyngeal squamous cell carcinoma. Taken together, there is no consensus opinion concerning the prognostic value of pre-treatment apoptosis frequency.

Paper 2

Estimating the clinical outcome of patients with squamous cell carcinoma in the head and neck is commonly based on tumour stage and localization since no reliable predictive tests have yet been applied to clinical use. Therefore, there is a great interest in tools predicting prognosis and tests predicting the therapeutic effects of new cancer drugs. In the present investigation, aspects of cell proliferation and apoptosis were studied in a relatively homogeneous group of oral cancers concerning tumour size and origin. The present investigation revealed that metallothionein (MT) was present in practically all tongue cancers. Evidence suggests a role for MTs in cancer development, treatment resistance and prognosis (Theocharis et al. 2004). MT overexpression is also a highly significant and independent factor for prognosis in malignant melanoma (Weinlich 2006) and predictive of worse patient outcome in breast cancer (Bay et al. 2006); it is also associated with resistance to anticancer drugs (Sunada et al. 2005).

In the present investigation, no significant correlation of MT to clinical outcome was found when the follow-up time was restricted to three years. In addition, the studied tumour group comprised the ones that generally have the best prognosis - tumours in the lowest stages. Both conditions might have influenced the results. Nakano et al. (2003) showed that MT mRNA levels were induced in cisplatin-resistant tongue squamous cell carcinoma cells after cisplatin treatment, but not in the cisplatin-sensitive tongue cancer cell line. Moreover, nuclear MT expression was found in cisplatin-resistant ovarian carcinoma cells and the nuclear staining pattern was specific for ovarian cancers of poor clinical outcome (Surowiak 2007). The p53 tumour suppressor protein has a DNA binding domain stabilized by a Zn ion, which is necessary for a functionally active configuration of wild-type p53 (Cho et al. 1994). Chelation of Zn (e.g., by MTs) disrupts the architecture of the p53 DNA binding domain, inducing the protein to adopt an immunological phenotype identical to that of many mutant p53 isoforms (Meplan et al. 2000). p53 was not studied in the present tongue cancer specimens, but the involvement of MT in p53 function is interesting. If cancer cells over-express MT, they may also induce a p53-null state in the presence of wt p53 and by that means, promote tumour growth and increase survival.

Both normal epithelium and cancerous lesions showed a similar staining pattern of MT in the present investigation. The peripheral expression of metallothionein may reflect the ongoing proliferative, rather than apoptotic, capacity in these areas. Interestingly, most proliferation activity (by means of Ki-67/MIB-1 staining) was observed in peripheral tumour cells and an opposite pattern was observed for the TUNEL-activity. These results indicate that MT may hinder tumour cells from entering apoptosis and thereby contribute to proliferation in these areas.

The Fas receptor was over-expressed in all tongue cancers whereas only half of the controls (normal squamous cell epithelium) stained positively in the present study. Recently, Fas expression was detected in 90% of esophageal squamous cell carcinomas, and a high expression was associated with better survival (Chan et al. 2006). Jackel et al. (2001) showed Fas expression in all studied laryngeal squamous cell carcinomas, but the positive staining had no impact on patient survival. Similar results were reported by Bayazit et al. (2000). Muraki et al. (2000) showed that two thirds of low stage (I and II) oral squamous cell carcinomas stained positive for Fas, and when assessed together with high stage tumours, the Fas-positive group had a better overall survival. In the present study, Fas expression was not correlated to patient outcome, but the inclusion of only stage I-II tongue cancers may have affected these results.

The difference between intracellular and membrane Fas staining was not taken into consideration in the present investigation. The receptors are functional only when anchored to the cell membrane. Still, intracellular receptors may play an important, active role in apoptosis

initiation. Bennett et al. (1998) showed that stimulation by p53 caused a rapid transport of receptors deposited in the Golgi apparatus to the cell membrane.

In the present study, a few tumour specimens stained positive for Bcl-2 and no significant correlation between the expression of Bcl-2 protein and clinical outcome was found. Several later studies on oral/oropharyngeal cancer have shown similar results where Bcl-2 overexpression has not been correlated to survival (Veneroni et al. 1997, Stoll et al. 2000, Yuen et al. 2002). In 2006, de Vicente et al. studied squamous cell carcinomas of the tongue with a majority of stage III-IV tumours, and found that patients with Bcl-2-negative tumours had longer survival. Similar results on oral cancer were also reported by Xie et al. (1999) and Popovic et al. (2007), who included mainly advanced tumours (stage III-IV). Recently, a large study on head and neck cancer patients showed that positivity in Bcl-2 together with MMP-9 was significantly related to the overall survival of patients (Smilek et al. 2006). Taken together, no uniform opinion on Bcl-2 as a prognostic marker in head and neck cancer exists. As discussed earlier, the biological relevance of Bcl-2 protein is highly dependent on the other members of the protein family. The Bcl-2/Bax expression ratio has been used more recently for taking into account both pro- and anti-apoptotic proteins in the assessment of apoptosis propensity. Nix et al. (2005) demonstrated that resistance to radiotherapy in early stage laryngeal cancers (T1-T2) was associated with high Bcl-2 and Bcl-XL expression and loss of Bax expression when pre-treatment biopsies were studied retrospectively. Furthermore, Tsuji et al. (2007) showed that oral cancer patients with high pre-therapeutic Bax expression in tumours had a good effect from cisplatin-based neoadjuvant chemotherapy when the surgical specimens were analysed. The overall survival rate in these "effective cases" was significantly higher than in "non-effective" cases.

Paper 3

Cell surface receptors, such as Fas, EGFR, and ICAM-1, are involved in control or progression of malignant tumors. Therapeutic drugs can potentially modulate the expression of these receptors and thus modulate the therapeutic efficacy of targeting drugs. Cisplatin, IFN-α, and 13-cisRA modulated the expression of Fas, EGFR, and ICAM-1 in oral squamous cell carcinoma cell lines, but affected receptor expression differently in the two studied cell lines. This is similar to results shown by Chen et al. (2000). The cisplatin effects were more uniform in comparison to IFN-α and 13-cisRA, which had varying effects on the receptors. The major effect of cisplatin was up-regulation of Fas expression. Similar results were reported by Iwase et al. (2003). However, Kuwahara et al. (2000) did not find any increase in Fas expression when cells were incubated with a ten times higher cisplatin concentration compared to the present study. The reason may be that the high concentration of cisplatin is more likely cytotoxic than modulating, as reported earlier in several toxicity studies (Kuwahara et al. 2000, Kinoshita et al. 2000, van Geelen et al. 2003). The platinum-based carboplatin, which is close to cisplatin, was shown by Mishima et al. (2003) to induce Fas-dependent apoptosis of tongue carcinoma cells earlier, and a specific antisense oligonucleotide against FADD abolished the effect.

Cisplatin resistance is a common obstacle in the treatment of HNSCC (Ferguson 1995). The resistance may be caused by cisplatin-induced phospatidylinositol 3-kinase/Akt pathway activation (Winograd-Katz and Lewitzki 2006). Takaoka et al. (2007) elegantly demonstrated how combining cisplatin with an anti-EGFR monoclonal antibody (C225) or an EGFR-selective tyrosine kinase inhibitor (AG1478) inhibited cell proliferation and induced apoptosis in a synergistic manner.

In the present study, the results from the immunohistochemical staining concerning Fas expression were not in accordance with those using the ELISA technique. Incubation with cisplatin enhanced the staining intensity in both cell lines, whereas the receptor amount was not

increased. When cells are grown as a monolayer on slides and directly stained, the cell membrane is intact. Staining of tissue sections, on the contrary, present a cross-section and therefore a better exposure of the cytoplasm. This may complicate the assessment of staining results on chamber slides since changes in staining intensity of the covering cell membrane may be falsely interpreted. Accordingly, a true increase in membrane staining may be assessed as a false increase of cytoplasm staining. In the present investigation, this confounding factor cannot be excluded. Furthermore, the staining procedure in the present investigation was optimised for detection of membrane-bound receptors; no detergent was applied to the staining procedure. Taken together, the enhanced Fas staining observed in the present thesis may be a result of intracellular movement of Fas receptors from the cytoplasm to the cell membrane without a total net increase of receptors. To be more precise with receptor assessment, supplementary investigations, such as confocal microscopy, PCR or Western blot, could be considered.

Both cell lines used in the present thesis were sensitive to apoptosis induction by the presence of an agonistic antibody (CH11), irrespective of p53 status, but only one of the cell lines, UT-SCC-24A, showed an additionally increased caspase-3 activity by cisplatin treatment. Iwase et al. (2003) reported a similar effect by a CH11 agonistic antibody on Fas-mediated activation of apoptosis after cisplatin treatment of squamous cell carcinoma cells. Low-dose cisplatin treatment has previously been shown to induce apoptosis after Fas stimulation in hepatic cancer cells with normal (wild-type) p53 (Müller et al. al 1998). The importance of normal p53 for this Fas-mediated apoptosis is also supported by studies on lung and prostate cancer cell lines (Li et al. 2003). The Fas-mediated cell death, however, cannot be solely dependent on a normal p53, which is shown in the present thesis, but a further enhanced apoptotic cell death caused by cisplatin may involve normal protein. p53 is a transcriptional regulator of the *cd95*(Fas) gene (Owen-Schaub et al. 1995) and the activated p53 can increase surface Fas expression by transport from the Golgi complex, thereby promoting enhanced apoptosis (Bennett et al. 1998). Other mechanisms, such as inhibition of FLIPs (death domain-like interleukin-1β-converting enzyme-inhibitory protein) by cisplatin, may be involved in regulation of the Fas-mediated apoptosis (Kim et al. 2003). An enhanced secretion of death receptors by chemotherapeutic drugs may contribute to resistance to cancer treatment. The secretion of Fas can theoretically protect cells from apoptosis if soluble receptors neutralize Fas ligand in the extracellular space before they reach membrane-bound receptors. This might explain why apoptosis was not increased in the cell line UT-SCC-20A.

The inhibitors of apoptosis proteins (IAPs) are a family of proteins that are able to regulate apoptosis when ectopically expressed in cells. They function to block cell death by binding to and inhibiting the action of caspases, and thus function downstream of both the intrinsic Bcl-2 family modulated and external death receptor-mediated pathways of cell death (Holcik et al. 2001). Survivin is the smallest member of the IAP protein family. It is absent or present at very low levels in normal adult tissue, but appears at increased levels in a wide variety of solid tumours and haematological malignancies acting as an inhibitor of apoptosis (Grossman and Altieri 2001). However, a bifunctional role of survivin has been reported recently since the protein also functions as a mitotic effector (reviewed in Stauber et al. 2007). Nitric oxide (Dash et al. 2007), members of Bcl-2 protein family (Yang et al. 2007, Oh et al. 2007), interleukin-4 (Rautajoki et al. 2007), and interleukin-10 (Bharhani et al. 2006) may be intervening factors in Fas-mediated apoptosis, but have not been specifically studied in the present investigation.

Paper 4

There is an expanding knowledge on the role of extracellular matrix, blood vasculature, inflammatory cells and, most of all, fibroblasts in cancer initiation and progression (Bhowmick et al. 2004, Smalley et al. 2005). The enhanced intratumoural proteolytic activity is also crucial for malignant behaviour of cancer since proteases play a major role in infiltrative growth, metastasis, and neo-angiogenesis (Stamenkovic 2003). Besides cancer cells, both stromal and immune cells secrete proteases and accordingly, are involved in tumour progress (Egeblad and Werb 2002). In the present study, both cell lines secreted MMP-1, MMP-2 and MMP-9 spontaneously. Earlier studies have shown that MMP expression in oral non-cancerous mucosa (epithelial dysplasias, lichen planus, and normal epithelial cells) is consistently low (Sutinen et al. 1998). The same authors also demonstrated that MMP-1 mRNA and MMP-2 mRNA expression has been detected mostly in fibroblasts of tumoural stroma in oral cancer samples (Sutinen et al. 1998). Later studies (Baker et al. 2006, Gorogh et al. 2006), however, also show MMP-1, MMP-2 and MMP-9 expression in oral cancer. Ruokolainen et al. (2006) showed a significantly better 5-year survival rate in patients with low pre-treatment serum MMP-9 levels compared to patients with high levels. Recently, Kwak et al. (2007) showed that the cyclooxygenase-2 (COX-2) inhibitor celecoxib inhibited MMP-2 and MMP-9 activity by 30%-40% in oral squamous cell carcinoma cells. Furthermore, clinically used epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) decreased both MMP-2 and MMP-9 enzyme activity by approximately 25%-30% in oral cancer cells (Lee 2007).

It is interesting to see that several effects other than the target specific may be seen when drug antagonists are used. In the present investigation, MMP-1 and MMP-9 secretion was affected by cytokines in cultures of both cell lines, but no significant effect was found on MMP-2 secretion. There is some evidence that TNF-α stimulates fibroblasts to increase MMP-2 mRNA levels, but no increase of enzyme secretion can be detected (Braundmeier and Nowak 2006). Furthermore, studies by Sutinen et al. (1998) and Zhang et al. (2006) indicate that fibroblast-derived MMP-2 (and also MT1-MMP) may have an important role in HNSCC tumour invasion.

The present results show for the first time a synergistic mechanism in squamous cell carcinoma cells, as MMP-1 secretion was synergistically up-regulated in both of the tested cell lines, and MMP-9 in UT-SCC-24A, after both IL-6 and sIL-6R were added. Interleukin-6 is an interesting cytokine in the context of head and neck cancer. Nitsch et al. (2007) recently demonstrated how head and neck cancers increased interleukin-6 secretion from tumour infiltrating immune cells facilitating escape from efficient immune responses and triggering its own malignant progression. Furthermore, tumour-associated macrophages secrete IL-6 in head and neck cancer tissue (Kross et al. 2007) and significantly elevated serum levels of IL-6 were detected in head and neck cancer patients (Hoffmann et al. 2007). Riedel et al. (2005) reported that IL-6 serum levels were elevated in the majority of HNSCC cancer patients and demonstrated correlation with tumour stage and lymph node status.

The results obtained in the present thesis concur well with those reported by Hanzawa et al. (2000), who showed that HGF increased MMP-1, -3 and -9 mRNA in an oral cancer cell line, but had no effect on MMP-2 mRNA. An increase of MMP-2 activity was reported by Bennett et al. (2000) after stimulation by HGF, as determined by zymography in oral SCC cell lines. Earlier, Lengyel et al. (1995) demonstrated that co-culture of a squamous cell carcinoma cell line with fibroblasts augmented matrix metalloproteinase-9 (MMP-9) expression and activity. A soluble factor produced by stromal cells was considered to be the most plausible explanation because fibroblast-conditioned medium had the same effect on cancer cells.

A clinical application of HGF assessment was recently published by Kim et al. (2007). They evaluated the correlation between serum HGF concentration and the progression of head and

neck squamous cell carcinoma. The patients´ HGF levels were significantly correlated to tumour stage progression and were higher than levels in healthy control subjects. Furthermore, the serum HGF levels decreased to normal 1 month after curative surgery and increased again in patients with recurrence of tumour. Also, the receptor for HGF, c-Met, has been reported to have an association with chemoresistance both in squamous cell carcinoma cell lines and head and neck cancer patients (Åkervall et al. 2006).

Mann et al. (1995) reported that TNF-alpha enhanced MMP-9 mRNA expression and proteinase activity in SCC. Over-expression of MMP-13 protein was observed in cutaneous SCC cells after TNF-alpha treatment, whereas no effect was found on MMP-1 production (Johansson et al, 2000). The present study supports these previous studies since TNF-alpha up-regulated MMP-9 secretion in both tumour cell lines. Lee et al. (2006) and Braundmeier and Nowak (2006) showed an enhanced MMP-1 secretion by TNF-alpha, which is also in accordance with results of the present thesis.

No specific spontaneous IL-6 secretion in the cell lines of the present study has been found earlier (personal communication). However, the assessment of the spontaneous secretion of cytokines from cancer cells should always be considered to assure that the possible spontaneous secretion of cytokines from cancer cells does not dominate over added cytokine. Pries et al. (2006) report high levels of secreted IL-6 and IL-8 in squamous cell carcinoma cultures. Serum proteins in the culture medium may also have an influence on the proteolytic activity; an abundant plasma protein α2-macroglobulin is a major inhibitor of MMPs (Sottrup-Jensen and Birkedal-Hansen 1989). In the present investigation, the occurrence of MMPs was therefore assessed in serum-free culture medium, but the presence of natural MMP inhibitors, TIMPs, was not studied.

The results from paper 3 and 4 can clearly demonstrate how different cell lines, albeit from similar origins, behave quite differently in their response to drugs and cytokines. These results reflect the complexity of cancer biology at a cellular level, something not taken into account in the current clinical praxis.

6 CONCLUSIONS

Patients with stage I acinic cell cancer showed a better prognosis than patients with stages II, III, and IV together. Ki-67 positivity seems to be an independent prognostic factor for survival in acinic cell cancer but Bcl-2 expression or spontaneous apoptosis frequency was not correlated with patient outcome. More efforts need to be put toward understanding the aspects of apoptosis regulation in malignant salivary gland tumours.

Metallothionein may hinder tumour cells from entering apoptosis and thereby contribute to proliferation of the areas with less differentiation where more MT positivity but less apoptosis was shown. The death receptor Fas was commonly expressed in tongue cancer cells, but not in normal oral mucosa. Metallothionein, Bcl-2, and Fas expression are not correlated with patient outcome in the studied group of tongue cancers.

Cisplatin, IFN-α, and 13-cisRA modulated the expression of Fas, EGFR, and ICAM-1 in oral squamous cell carcinoma cell lines, but the drugs affected receptor expression differently. The low-toxic dose of a widely used chemotherapeutic drug, cisplatin, increases the amount of death receptor Fas in both cell lines and moreover, cisplatin seems to increase the receptor-mediated cell death.

The oral cancer cells secrete tissue degrading matrix metalloproteinase-1, -2, and -9, and the amount of secreted MMP-1 and MMP-9 was enhanced when cytokines were added to the cell cultures. The cytokine-induced enzyme secretion and the synergistic effect of two cytokines, which was demonstrated in the present investigation, implies that more research needs to be done on the tumour micro-environment since the studied cytokines may be secreted by non-cancerous fibroblasts and immune cells.

7 FUTURE RESEARCH

The salivary gland tumours are a heterogeneous group of neoplasms that are commonly treated by surgery, and the treatment is combined with radiotherapy in selected cases. Different aspects of tumour growth have not been studied to a great extent in malignant salivary gland tumours mainly because of the rarity of separate sub-groups defined by histo-pathological classification. Systemic chemotherapy plays only a palliative role in the contemporary treatment. From a clinician's perspective, radiosensitisation and development of new therapeutic drugs are two major areas asked for in the research field of salivary gland malignancies. To have the possibility to apply predictive factors in clinical use would contribute to more individual therapy planning. At a cellular level, the mechanisms controlling growth of these tumours, including apoptosis and proliferation, are subjects for feasible future studies. Also, the role of tumour surrounding normal cells both in the development of the cancer and in facilitating/inhibition of the tumour growth needs to be further investigated. Ultimately, studying genetic alterations that lead to tumorigenesis of the salivary glands may widen the therapeutic options. For that purpose, collaboration between several cancer clinics is needed to obtain a sufficient amount of tumour specimens in all the histopathological subgroups of salivary gland tumours.

The surgical and radiotherapeutic treatment of squamous cell carcinoma in the head and neck has improved remarkably during the last few decades, but the third arm in treatment, chemotherapy, has not evolved at the same rate. Therefore, there is a need to improve both the effect of available chemotherapeutic drugs and to develop new drugs that are effective in head and neck cancer. The cellular mechanisms underlying particularly chemoresistance, but also radioresistance, are conceivable subjects for further investigations. In this context, a comprehensive study of apoptosis in head and neck cancers, which will be a huge research assignment, would certainly give new insights into the treatment obstacles on a cellular level. A realistic way to approach this problem will be to study one drug or treatment modality at a time. In order to develop new drugs, both tumour micro-environmental factors, including matrix metalloproteinases and cytokines, apoptosis mechanisms and cell proliferation potential, are in focus. A major problem today is the enormous quantity of research data that need to be sorted and evaluated. Larger investigations, which can verify the results of small-scale *in vitro*- and *in vivo*- studies, are still lacking which hinder the direct application of research results to clinical practise.

8 REFERENCES

Abdel-Mageed AB, Agrawal KC. Activation of nuclear factor κB: potential role in metallothionein-mediated mitogenic response. *Cancer Res* 1997;58:2335-2338

Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? *Biochem Pharmacol* 2006;72:1605-1621

Algeciras-Schimnich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK, Peter ME. Molecular ordering of the initial signalling events of CD95. *Mol Cell Biol* 2002;22:207-220

Apostolova MD, Cherian MG. Nuclear localization of metalothionein during cell proliferation and differentiation. *Cell Mol Biol* 2000;46:347-356

Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nature Reviews Cancer* 2002;2:420-430

Atabey N, Gao Y, Yao ZJ, Breckenridge D, Soon L, Soriano JV, Burke TR Jr, Bottaro DP. Potent blockade of hepatocyte growth factor-stimulated cell motility, matrix invasion and branching morphogenesis by antagonists of Grb2 Src homology domain interactions. *J Biol Chem* 2001;276:14308-14314,

Azemar M, Schmidt M, Arlt F, Kennel P, Brandt B, Papadimitriou A, Groner B, Wels W. Recombinant antibody toxins specific for ErbB2 and EGF receptor inhibit the in vitro growth of human head and neck cancer cells and cause rapid tumour regression in vivo. *Int J Cancer* 2000;86:269-275

Badros AZ, Goloubeva O, Rapoport AP, Ratterree B, Gahres N, Meisenberg B,Takebe N, Heyman M, Zwiebel J, Streicher H, Gocke CD, Tomic D, Flaws JA, Zhang B, Fenton RG. Phase II study of G3139, a Bcl-2 antisense oligonucleotide, in combination with dexamethasone and thalidomide in relapsed multiple myeloma patients. *J Clin Oncol* 2005;23:4089-4099.

Baker EA, Leaper DJ, Hayter JP, Dickenson AJ. The matrix metalloproteinase system in oral squamous cell carcinoma. *Br J Oral Maxillofac Surg* 2006;44:482-486

Balint E E, Vousden KH. Activation and activities of the p53 tumour suppressor protein. *Br J Cancer* 2001;85:1813-1823

Bancroft CC, Chen Z, Yeh J, Sunwoo JB, Yeh NT, Jackson S, Jackson C, Van Waes C. Effects of pharmacologic antagonists of epidermal growth factor receptor, PI3K and MEK signal kinases on NF-kappaB and AP-1 activation and IL-8 and VEGF expression in human head and neck squamous cell carcinoma lines. *Int J Cancer* 2002;99:538-548

Barillé S, Collette M, Thabard W, Bleunven C, Bataille R, Amiot M. Soluble IL-6Rα upregulated IL-6, MMP-1 and MMP-2 secretion in bone marrow stromal cells. *Cytokine* 2000;12:1426-1429

Bates S, Vousden KH. Mechanisms of p53-mediated apoptosis. *Cell Mol Life Sci* 1999;55:28-37

Bay BH, Jin R, Huang J, Tan PH. Metallothionein as a prognostic biomarker in breast cancer. *Exp Biol Med* 2006;231:1516-1521

Bayazit Y, Mumbuc S, Ucak R, Kanlikama M, Bakir K. Significance of Fas protein in squamous cell carcinoma of the larynx. *Acta Otolaryngol* 2000;120:557-561

Beacham DA, Cukierman E. Stromagenesis: the changing face of fibroblastic microenvironments during tumor progression. *Semin Cancer Biol* 2005;15:329-341

Bellacosa A, Almadori G, Cavallo S Cadoni G, Galli J, Ferrandina G, Scambia G, Neri G. Cyclin D1 gene amplification in human laryngeal squamous cell carcinomas: prognostic significance and clinical implication. *Clin Cancer Res* 1996;2:175-180

Ben-Izhak O, Laster Z, Araidy S, Nagler RM. TUNEL – an efficient prognosis predictor of salivary malignancies. *Br J Cancer 2007*;96:1101-1106

Benner SE, Pajak TF, Lippman SM, Earley C, Hong WK. Prevention of second primary tumors with isotretinoin in patients with squamous cell carcinoma of the head and neck: long-term follow-up. *J Natl Cancer Inst* 1994;86:140-141

Bennett M, Macdonald K, Chan S-W, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* 1998;282: 290-293

Bennett JH, Morgan MJ, Whawell SA, Atkin P, Roblin P, Furness J, Speight PM. Metalloproteinase expression in normal and malignant oral keratinocytes: stimulation of MMP-2 and MMP-9 by scatter factor. *Eur J Oral Sci* 2000;108:281-291

Bharhani MS, Borojevic R, Basak S, Ho E, Zhou P, Croitoru K. IL-10 protects mouse intestinal epithelial cells from Fas-induced apoptosis via modulating Fas expression and altering caspase-8 and FLIP expression. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G820-829.

Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* 2004;432:332-337

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 1993;4:197-250

Biron CA. Role of early cytokines, including alpha and beta interferons (IFN alpha/beta), in innate and adaptive immune responses to viral infections. *J Immunol* 1998;10:383-390

Biron CA. Interferons alpha and beta as immune regulators – a new look. *Immunity* 2001;14:661-664

Bogusiewicz M, Stryjecka-Zimmer M, Szymanski M, Rechberger T, Golabek W. Activity of matrix metalloproteinases-2 and -9 in advanced laryngeal cancer. *Otolaryngol Head Neck Surg* 2003;128:132-136

Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R,Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med* 2006;354:567-578.

Bossi G, Sacchi A. Restoration of wild-type p53 function in human cancer: relevance for tumor therapy. *Head Neck* 2007;29:272-284

Bova RJ, Quinn DI, Nankervis JS, Cole IE, Sheridan BF, Jensen MJ, Morgan GJ, Hughes CJ, Sutherland RL. Cyclin D1 and p16INK4A expression predict reduced survival in carcinoma of the anterior tongue. *Clin Cancer Res* 1999;5:2810-2819

Braundmeier AG, Nowak RA. Cytokines regulate matrix metalloproteinases in human uterine endometrial fibroblast cells through a mechanism that does not involve increases in extracellular matrix metalloproteinase inducer. *Am J Reprod Immunol* 2006;56:201-214.

Bukowski R, Ernstoff MS, Gore ME, Nemunaitis JJ, Amato R, Gupta SK, Tendler CL. Pegylated interferon alfa-2b treatment for patients with solid tumours: a phase I/II study. *J Clin Oncol* 2002;20:3841-3849

Burgess AW, Cho HS, Eigenbrot C, Ferguson KM, Garrett TP, Leahy DJ, Lemmon MA, Sliwkowski MX, Ward CW, Yokoyama S. An open-and-shut case? Recent insights into activation of EGF/Erbb receptors. *Mol Cell* 2003;12:541-552

Burtness B, Goldwasser MA, Flood W, Mattar B, Forastiere AA. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an eastern Cooperative Oncology Group study. *J Clin Oncol* 2005;23:8646-8654

Chan KW, Lee PY, Lam AK, Law S, Wong J, Srivastava G. Clinical relevance of Fas expression in oesophageal squamous cell carcinoma. *J Clin Pathol* 2006;59:101-104

Chang DW, Xing Z, Pan Y Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X. c-FLIPL is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J* 2002;21:3704-3714

Chen Z, Seimiya H, Naito M, Mashima T, Kizaki A, Dan S, Imaizumi M, Ichijo H, Miyazono K, Tsuruo T. ASK1 mediates apoptotic cell death induced by genotoxic stress. *Oncogene* 1999;18:173-180

Chen Z, Ke LD, Yuan XH, Adler-Storthz K. Correlation of cisplatin sensitivity with differential alteration of EGFR expression in head and neck cancer cells. *Anticancer Res* 2000;20:899-902

Cherian MG. Jayasurya A, Bay BH. Metallothioneins in human tumors and potential roles in carcinogenesis. *Mut Res* 2003;533:201-209

Chiou SK, Rao L, White E. Bcl-2 blocks p53 –dependent apoptosis. *Mol Cell Biol* 1994;14:2556-2563

Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor –DNA complex: understanding tumorigenic mutations. *Science* 1994;265:346-355

Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997;326:1-16

Cory S, Huang DC, Adams JM. The bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003;22:8590-8607

Costes V, Liautard J, Picot MC, Robert M, Lequeux N, Brochier J, Baldet P,Rossi JF. Expression of the interleukin 6 receptor in primary renal cell carcinoma. *J Clin Pathol* 1997;50:835-840

Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002;295:2387-2392

Dash PR, McCormick J, Thomson MJ, Johnstone AP, Cartwright JE, Whitley GS. Fas ligand-induced apoptosis is regulated by nitric oxide through the inhibition of Fas receptor clustering and the nitrosylation of protein kinase Cepsilon. *Exp Cell Res* 2007;313:3421-3431

DeClerck YA, Mercurio AM, Stack MS, Chapman HA, Zutter MM, Muschel RJ, Raz A, Matrisian LM, Sloane BF, Noel A, Hendrix MJ, Coussens L, Padarathsingh M. Proteases, extracellular matrix, and cancer: a workshop of the path B study section. *Am J Pathol* 2004;164:1131-1139

de Vicente JC, Olay S, Lequerica-Fernandez P, Sanchez-Mayoral J, Junquera LM, Fresno MF. Expression of Bcl-2 but not Bax has a prognostic significance in tongue carcinoma. *J Oral Pathol Med* 2006;35:140-145

Deveraux QL, Reed JC. IAP family proteins – suppressors of apoptosis. *Genes Dev* 1999;13:239-252

de Vries N, Snow GB. Relationships of vitamins A and E and beta-carotene serum levels to head and neck cancer patients with and without second primary tumors. *Eur Arch Otorhinolaryngol* 1990;247:368-370

Dunne AA, Sesterhenn A, Gerisch A, Teymoortash A, Kuropkat C, Werner JA. Expression of MMP-2, -9 and –13 in cell lines and fresh biopsies of squamous cell carcinomas of upper aerodigestive tract. *Anticancer Res* 2003;23:2233-2240

Dvorak H. Tumours: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Eng J Med* 1986;315:1650-1659

Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cell 2, 1990;275-80

Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nature Rev* 2002;2:161-174

Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, Collins VP, James CD. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. *Oncogene* 1994;9:2313-2320

El-Deiry WS. Insights into cancer therapeutic design based on p53 and TRAIL receptor signalling. *Cell Death Diff* 2001;8:1066-1075

Esposito V, Groeger AM, De Luca L, Di Marino M, Santini D, Marchei P, Baldi F, Wolner E, Baldi A. Expression of surface protein receptors in lung cancer. *Anticancer Res* 2002;22:4039-4043.

Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-767

Feliciani C, Gupta AK, Sauder DN. Keratinocytes and cytokine/growth factors. *Crit Rev Oral Biol Med* 1996;7:300-318

Ferguson PJ. Mechanisms of resistance of human tumours to anticancer drugs of the platinum family: a review. *J Otolaryngol* 1995;24:242-252

Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. *N Engl J Med* 2001;345:1890-1900

Gaiotti D, Chung J, Iglesias M, Nees M, Baker PD, Evans CH, Woodworth CD. Tumor necrosis factor-alpha promotes human papillomavirus (HPV) E6/E7 RNA expression and cyclin-dependent kinase activity in HPV-immortalized keratinocytes by a ras-dependent pathway. *Mol Carcinog* 2000;27:97-109

Gallo O, Boddi V, Calzolari A, Simonetti L, Trovati M, Bianchi S. bcl-2 protein expression correlates with recurrence and survival in early stage head and neck cancer treated by radiotherapy. *Clin Cancer Res* 1996;2:261-267

Gao M, Fan S, Goldberg ID, Laterra J, Kitsis RN, Rosen EM. Hepatocyte growth factor/scatter factor blocks the mitochondrial pathway of apoptosis signaling in breast cancer cells. *J Biol Chem* 2001;276:47257-47265.

Gastman BR, Atarshi Y, Reichert TE, Saito T, Balkir L, Rabinowich H, Whiteside TL. Fas ligand is expressed on human squamous cell carcinomas of the head and neck, and it promotes apoptosis of T lymphocytes. *Cancer Res* 1999;59:5356-5364

Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labelling of nuclear fragmentation. *J Cell Biol* 1992;119:493-501

Gerdes J, Becker MH, Key G, Cattoretti G. Immunohistological detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. *J Pathol* 1992;174:1685-1687

Gollin SM. Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease. *Head Neck* 2001;23:238-253

Gorogh T, Beier UH, Baumken J, Meyer JE, Hoffmann M, Gottschlich S, Maune S. Metalloproteinases and their inhibitors: influence on tumor invasiveness and metastasis formation in head and neck squamous cell carcinomas. *Head Neck* 2006;28:31-39

Grossman D and Altieri DC. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. *Cancer Metastasis Rev* 2001;20:3-11

Guillaud P, du Manoir S, Seigneurin D. Quantification and topographical description of Ki-67 antibody labelling during the cell cycle of normal fibroblastic (MRC-5) and mammary tumour cell lines (MCF-7). *Anal Cell Pathol* 1989;1:25-39

Gudas LJ, Sporn MB, Roberts AB. Cellular biology and biochemistry of the retinoids. In: Sporn MB, Robetrs AB, Goodman DS. The retinoids. Biology, chemistry and medicine 2nd ed. New York: Raven Press, 1994. p. 443-520

Gupta V, Jani JP, Emerson EO, Xu BH, Scalamogna D, Xia H, Katoh A, Zaren HA, Singh SV. Modualtion of cisplatin sensitivity and accumulation by interferon α-2a in human squamous cell carcinoma cell lines. Int J Cancer 1995, 63:41922

Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70

Hanzawa M, Shindoh M, Higashino F, Yasuda M, Inoue N, Hida K, Ono M, Kohgo T, Nakamura M, Notani K, Fukuda H, Totsuka Y, Yoshida K, Fujinaga K. Hepatocyte growth factor upregulates E1AF that induces oral squamous cell carcinoma cell invasion by activating matrix metalloproteinase genes. *Carcinogenesis* 2000;21:1079-1085

Harari PM, Huang SM. Head and neck cancer as a clinical model for molecular targeting of therapy: combining EGFR blockade with radiation. *Int J Radiat Oncol Biol Phys* 2001;49:427-433

Harari PM, Allen GW, Bonner JA. Biology of interactions: antiepidermal growth factor receptor agents. *J Clin Oncol* 2007;25:4057-4065

Harris HW, Gill TJ. Expression of class I transplantation antigens. *Transplantation* 1986;42:109-117

Hauser U, Balz V, Carey TE, Grénman R, van Lierop A, Scheckenbach K, Bier H. Reliable detection of p53 aberrations in squamous cell carcinomas of the head and neck requires transcript analysis of the entire coding region. *Head Neck* 2002;24:868-873

Hishikawa Y, Abe S, Kinugasa S, Yoshimura H, Monden N, Igarashi M, Tachibana M, Nagasue N. Overexpression of metallothionein correlate with chemoresistance to cisplatin and prognosis in esophageal cancer. *Oncology* 1997,54:342-347

Hoffmann S, Rockenstein A, Ramaswamy A, Celik I, Wunderlich A, Lingelbach S, Hofbauer LC, Zielke A. Retinoic acid inhibits angiogenesis and tumor growth of thyroid cancer cells. *Mol Cell Endocrinol* 2007;264:74-81.

Hoffmann TK, Sonkoly E, Homey B, Scheckenbach K, Gwosdz C, Bas M, Chaker A, Schirlau K, Whiteside TL. Aberrant cytokine expression in serum of patients with adenoid cystic carcinoma and squamous cell carcinoma of the head and neck. *Head Neck* 2007;29:472-478.

Hojilla CV, Mohammed FF, Khokha R. Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. *Br J Cancer* 2003;89:1817-1821

Holcik M, Gibson H, Korneluk RG. XIAP: the guardian angel. *Natl Rev Mol Cell Biol* 2001;2:550-556

Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;253:49-53

Holzmann B, Lehmann JM, Ziegler-Heitbrock L, Funke I, Riethmuller G and Johnson JP. Glycoprotein P3.58, associated with tumour progression in malignant melanoma, is a novel leucocyte activation antigen. *Int J Cancer* 1988,41:542-47

Hong WK, Endicott J, Itri LM, Doos W, Batsakis JG, Bell R, Fofonoff S, Byers R, Atkinson EN, Vaughan C, et al. 13-cis-retinoic acid in the treatment of oral leukoplakia. *N Engl J Med* 1986;315:1501-1505

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, et al. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1990;323:795-801

Horiuchi S, Koyanagi Y, Zhou Y, Miyamoto H, Tanaka Y, Waki M, Matsumoto A, Yamamoto M, Yamamoto N. Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism. *Eur J Immunol* 1994;24:1945-1948

Houston A, O'Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. *Curr Opin Pharmacol* 2004;4:321-326

Huang S, Mills L, Mian B Tellez C, McCarty M, Yang XD, Gudas JM, Bar-Eli M. Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibits angiogenesis, tumour growth, and metastasis of human melanoma. *Am J Pathol* 2002;161:125-34

Huh CG, Factor VM, Sanchez A, Uchida K, Conner EA, Thorgeirsson SS. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc Natl Acad Sci U S A* 2004;101:4477-4482

Ikebe T, Shinohara M, Takeuchi H, Beppu M, Kurahara S, Nakamura S, Shirasuna K. Gelatinolytic activity of matrix metalloproteinase in tumour tissues correlates with invasiveness in oral cancer. *Clin Exp Metastasis* 1999;17:315-323

Imanishi Y, Fujii M, Tokumaru Y, Tomita T, Kanke M, Kanzaki J, Kameyama K, Otani Y, Sato H. Clinical significance of expression of membrane type 1 matrix metalloproteinase and metalloproteinase-2 human head and neck squamous cell carcinoma. *Human Pathol* 2000;31:895-904

Ishikawa S, Takenaka K, Yanagihara K, Miyahara R, Kawano Y, Otake Y, Hasegawa S, Wada H, Tanaka F. Matrix metalloproteinase-2 status in stromal fibroblasts, not in tumor cells, is a significant prognostic factor in non-small-cell lung cancer. *Clin Cancer Res* 2004;10:6579-6585

Ivanov VN, Lopez Bergami P, Maulit G, Sato TA, Sassoon D, Ronai Z. FAP-1 association with Fas (Apo-1) inhibits Fas expression on the cell surface. *Mol Cell Biol* 2003;23:3623-3635

Iwase M, Watanabe H, Kondo G, Ohashi M, Nagumo M. Enhanced susceptibility of oral squamous cell carcinoma cell lines to Fas-mediated apoptosis by cisplatin and 5-fluorouracil. *Int J Cancer* 2003;106,619-625

Jackel MC, Mitteldorf C, Schweyer S, Fuzesi L. Clinical relevance of Fas (APO-1/CD95) expression in laryngeal squamous cell carcinoma. *Head Neck* 2001;23:646-652

Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res* 2000;60:184-190

Johansson N, Airola K, Grenman R, Kariniemi AL, Saarialho-Kere U, Kahari VM. Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. *Am J Pathol* 1997;151:499-508

Johansson N, Ala-aho R, Uitto V, Grenman R, Fusenig NE, Lopez-Otin C, Kahari VM. Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase. *J Cell Sci* 2000;113:227-235

Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomen with wide ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-256

Khuri FR, Lee JJ, Lippman SM, Kim ES, Cooper JS, Benner SE, Winn R, Pajak TF, Williams B, Shenouda G, Hodson I, Fu K, Shin DM, Vokes EE, Feng L, Goepfert H, Hong WK. Randomized phase III trial of low-dose isotretinoin for prevention of second primary tumors in stage I and II head and neck cancer patients. *J Natl Cancer Inst* 2006;98:441-450.

Kim JH, Ajaz M, Lokshin A, Lee YJ. Role of antiapoptotic proteins in tumor necrosis factor-related apoptosis-inducing ligand and cisplatin-augmented apoptosis. *Clin Cancer Res* 2003;9:3134-3141

Kim CH, Lee JS, Kang SO, Bae JH, Hong SP, Kahng H. Serum hepatocyte growth factor as a marker of tumor activity in head and neck squamous cell carcinoma. *Oral Oncol* 2007;43:1021-1025

Kinoshita H, Yoshikawa H, Shiiki K, Hamada Y, Nakajima Y, Tasaka K. Cisplatin (CDDP) sensitizes human osteosarcoma cell to Fas/CD95-mediated apoptosis by down-regulating FLIP-L expression. *Int J Cancer* 2000;88:986-991

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins from a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 1995;14:5579-5588

Kitadai Y, Takahashi Y, Haruma K, Naka K, Sumii K, Yokozaki H, Yasui W, Mukaida N, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice. *Br J Cancer* 1999;81:647-653

Kitson J, Raven T, Jiang Y-P, Goeddel DV, Giles KM, Pun K-T, Grinham CJ, Brown R and Farrow SN. A death-domain-containing receptor that mediates apopotosis. *Nature* 1996;384:372-375 OBS!! PLATSBYTE med Kitadai!!

Kleinerman ES, Zwelling LA. Effects of cisplatin, bleomycin and DTIC on immune function in vitro and in vivo. In: Mitchell MS, Fahey JL, editors. Immune suppression nad modulation. Clinics in Immunology and Allergy, vol 4, no 2. London: Saunders; 1984, pp 279-294

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 1992;258:1798-1801

Korsmeyer SJ. Regulators of cell death. *Trends Genet* 1995;11:101-105

Kroemer G, Petit P, Zamzami N, Vayssiere JL, Mignotte B. The biochemistry of programmed cell death. *FASEB J* 1995 ;9:1277-1287

Kross KW, Heimdal JH, Olsnes C, Olofson J, Aarstad HJ. Tumour-associated macrophages secrete IL-6 and MCP-1 in head and neck squamous cell carcinoma tissue. *Acta Otolaryngol* 2007;127:532-539.

Kune GA, Kune S, Field B, Watson LF, Cleland H, Merenstein D, Vitetta L. Oral and pharyngeal cancer, diet, smoking, alcohol, and serum vitamin A and beta-carotene levels: a case-control study in men. *Nutr Cancer* 1993;20:61-70

Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? *Histol Histopathol* 2002;17:599-621

Kurahara S, Shinohara M, Ikebe T, Nakamura S, Beppu M, Hiraki A, Takeuchi H, Shirasuna K. Expression of MMPs, MT-MMP and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumour invasion and metastasis. *Head Neck* 1999;21:627-638

Kuwahara D, Tsutsumi K, Kobayashi T, Hasunuma T, Nishioka K. Caspase-9 regulates cisplatin-induced apoptosis in human head and neck squamous cell carcinoma cells. *Cancer Lett* 2000;148:65-71

Kwak YE, Jeon NK, Kim J, Lee EJ. The cyclooxygenase-2 selective inhibitor celecoxib suppresses proliferation and invasiveness in the human oral squamous carcinoma.*Ann N Y Acad Sci* 2007;1095:99-112

Kägi JHR. Overview of metallothionein. *Methods Enzymology* 1991;205:613-626

LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* 1998;17:3247-3259

Lang S, Whiteside TL, Lebeau A, Zeidler R, Mack B, Wollenberg B. Impairment of T-cell activation in head and neck cancer in situ and in vitro. *Arch Otolaryngol Head Neck Surg* 1999;125:82-88

Lansdorf CD, Grénman R, Bier H, Somers KD, Kim S-Y, Whiteside TL, Clayman GL, Welkoborsky H-J, Carey TE. Head and Neck Cancers. In: Human Cell Culture Vol 2, Cancer Cell Lines Part 2. Masters J and Palsson B (eds). Kluwer Academic Press: Dordrecht (Holland), 1999, pp 185-255

Lebwohl D, Canetta R. Clinical development of platinum complexes in cancer therapy: a historical perspective and an update. *Eur J Cancer* 1998;34:1522-1534

Lefebvre JL. Current clinical outcomes demand new treatment options for SCCHN. *Annal Oncol* 2005;16:vi7-vi12

Lee EJ, Whang JH, Jeon NK, Kim J. The epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) suppresses proliferation and invasion of human oral squamous carcinoma cells via p53 independent and MMP, uPAR dependent mechanism. *Ann N Y Acad Sci* 2007;1095:113-128

Lee J, Jung E, Lee J, Huh S, Hwang CH, Lee HY, Kim EJ, Cheon JM, Hyun CG, Kim YS, Park D. Emodin inhibits TNF alpha-induced MMP-1 expression through suppression of activator protein-1 (AP-1). *Life Sci* 2006;79:2480-2485

Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature* 1987;329:630-632

Leivo I, Jee KJ, Heikinheimo K, Laine M, Ollila J, Nagy B, Knuutila S. Characterization of gene expression in major types of salivary gland carcinomas with epithelial differentiation. *Cancer Genet Cytogenet* 2005;156:104-113

Lengyel E, Gum R, Juarez J, Clayman G, Seiki M, Sato H, Boyd D. Induction of M_r92,000 type IV collagenase expression in a squamous cell carcinoma cell line by fibroblasts. *Cancer Res* 1995;55:963-967

Levi E, Fridman R, Miao HQ, Ma YS, Yayon A, Vlodavsky I. Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast grwth factor receptor 1. *Proc Natl Acad Sci USA* 1996;93:7069-7074

Leyshon-Sorland K, Morkrid L, Rugstad HE. Metallothionein: a protein conferring resistance in vitro to tumor necrosis factor. *Cancer Res* 1993;53:4874-4880

Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998;94:491-501

Li Y, Raffo AJ, Drew L, Mao Y, Tran A, Petrylak DP, Fine RL. Fas-mediated apoptosis is dependent on wild-type p53 status in human cancer cells expressing a temperature-sensitive p53 mutant alanine-143. *Cancer Res* 2003; 63:1527-1533

Lim JJ, Kang S, Lee MR, Pai HK, Yoon HJ, Lee JI, Hong SP, Lim CY. Expression of vascular endothelial growth factor in salivary gland carcinomas and its relation to p53, Ki-67 and prognosis. *J Oral Pathol Med* 2003;32:552-561

Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L, Hung MC. Nuclear localization of EGFR receptor and its potential new role in as a transcription factor. *Nat Cell Biol* 2001;3:802-808

Lisby S, Ralfkiaer E, Rothlein R, Vejlsgaard GL. Intercellular adhesion molecule-1 (ICAM-1) expression correlated to inflammation. *Br J Dermatol* 1989;120:479-484

List K, Szabo R, Molinolo A, Sriuranpong V, Redeye V, Murdock T, Burke B, Nielsen BS, Gutkind JS, Bugge TH. Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev* 2005;19:1934-1950

Liu M, Lawson G, Delos M Jamart J, Ide C, Coche E, Weynand B, Desuter G,Hamoir M, Remacle M, Marbaix E. Predictive value of the fraction of cancer cells immunolabeled for proliferating cell nuclear cell antigen or Ki67 in biopsies of head and neck carcinomas to identify lymph node metastasis: comparison with clinical and radiological examinations. *Head Neck* 2003;25:280-288

Liu Y, Sun AM, Dworkin LD. Hepatocyte growth factor protects renal epithelial cells from apoptotic cell death. *Biochem Biophys Res Commun* 1998;246:821-826.

Lothaire P, de Azambuja E, Dequanter D, Lalami Y, Sotiriou C, Andry G, Castro G Jr, Awada A. Molecular markers of head and neck squamous cell carcinoma: promising signs in need of prospective evaluation. *Head Neck* 2006;28:256-269.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951;193:265-275

Lu C, Vickers MF, Kerbel RS. Interleukin 6: a fibroblast-derived growth inhibitor of human melanoma cells from early but not advanced stages of tumor progression. *Proc Natl Acad Sci USA* 1992;89:9215-9219

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998;94:481-490

Luukkaa H, Klemi P, Leivo I, Vahlberg T, Grénman R. Prognostic significance of Ki-67 and p53 as tumor markers in salivary gland malignancies in Finland: an evaluation of 212 cases. *Acta Oncol* 2006;45:669-675

MacDougall JR, Matrisian LM. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. *Cancer Metast Rev* 1995;14:351-362

MacEwan DJ. TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal* 2002;14:477-492

Maguire HCJ, Ettore VL. Enhancement of dinitrochlorobenzene (DNCB) contact sensitization by cyclophosphamide in guinea pigs. *J Invest Dermatol* 1967;48:39-43

Malmberg KJ, Ljunggren HG. Escape from immune- and nonimmune-mediated tumor surveillance. *Semin Cancer Biol* 2006;16:16-31

Mann EA, Hibbs MS, Spiro JD, Bowik C, Wang XZ, Clawson M, Chen LL. Cytokine regulation of gelatinase production by head and neck squamous cell carcinoma: the role of tumor necrosis factor-alpha. *Ann Otol Rhinol Laryngol* 1995;104:203-209

Mannello F, Gazzanelli G. Tissue inhibitors of metalloproteinases and programmed cell death: conundrums, controversies and potential implications. *Apoptosis* 2001,6:479-482

Margolin K, Synold TW, Lara P, Frankel P, Lacey SF, Quinn DI, Baratta T, Dutcher JP, Xi B, Diamond DJ, Gandara DR. Oblimersen and alpha-interferon in metastatic renal cancer: a phase II study of the California Cancer Consortium. *J Cancer Res Clin Oncol* 2007;133:705-711

Matsumiya T, Imaizumi T, Yoshida H, Kimura H, Satoh K. Cisplatin inhibits the expression of X-chromosome-linked inhibitor of apoptosis protein in an oral carcinoma cell line. *Oral Oncol* 2001;37:296-300

Matsuoka H, Eura M, Chikamatsu K, Nakano K, Kanzaki Y, Masuyama K, Ishikawa T. Low doses of anticancer drugs increase susceptibility of tumor cells to lysis by autologous killer cells. *Anticancer Res* 1995;15:87-92

Melendez ND, Smoller BR, Morgan M. VCAM (CD-106) and ICAM (CD-54) adhesion molecules distinguish keratoacanthomas from cutaneous squamous cell carcinomas. *Mod Pathol* 2003;16:8-13

Meplan C, Richard MJ, Hainaut P. Metalloregulation of the tumor suppressor protein p53: zinz mediates the renaturation of p53 after exposure to metal chelators *in vitro* and in intact cells. *Oncogene* 2000;19:5227-5236

Micheau O, Solary E, Hammann A, Martin F, Dimanche-Boitrel MT. Sensitization of cancer cells treated with cytotoxic drugs to Fas-mediated cytotoxicity. *J Natl Cancer Inst* 1997;89:783-789.

Milatovich A, Song K, Heller RA, Francke U. Tumour necrosis factor receptor genes, TNFR1and TNFR2, on human chromosomes 12 and 1. *Somat Cell Mol Genet* 1991;17:519-523

Mishima K, Nariai Y, Yoshimura Y. Carboplatin induces Fas (APO-1/CD95)-dependent apoptosis of human tongue carcinoma cells: sensitization for apoptosis by upregulation of FADD expression. *Int J Cancer* 2003;105:593-600

Mita MM, Ochoa L, Rowinsky EK, Kuhn J, Schwartz G, Hammond LA, Patnaik A, Yeh IT, Izbicka E, Berg K, Tolcher AW. A phase I, pharmacokinetic and biologic correlative study of oblimersen sodium (Genasense, G3139) and irinotecan in patients with metastatic colorectal cancer. *Ann Oncol* 2006;17:313-321

Mitsiades N, Poulaki V, Tseleni-Balafouta S, Koutras DA, Stamenkovic I. Thyroid carcinoma cells are resistant to Fas-mediated apoptosis but sensitive to tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* 2000;60:4122-4129

Miyamoto R, Uzawa N, Nagaoka S, Hirata Y, Amagasa T. Prognostic significance of cyclin D1 amplification and overexpression in oral squamous cell carcinomas. *Oral Oncol* 2003;39:610-618.

Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* 2001;61:4744-4749

Morello S, Olivero M, Aimetti M, Bernardi M, Berrone S, Di Renzo MF, Giordano S. MET receptor is overexpressed but not mutated in oral squamous cell carcinomas. *J Cell Physiol* 2001;189:285-290

Mullberg J, Schooltink H, Stoyan T, Gunther M, Graeve L, Buse G, Mackiewicz A, Heinrich PC, Rose-John S. The soluble interleukin-6 receptor is generated by shedding. *Eur J Immunol* 1993;23:473-480

Mullberg J, Oberthur W, Lottspeich F, Mehl E, Dittrich E, Graeve L, Heinrich PC, Rose-John S. The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J Immunol* 1994;152:4958-4968

Muraki Y, Tateishi A, Seta C, Fukuda J, Haneji T, Oya R, Ikemura K, Kobayashi N. Fas antigen expression and outcome of oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* 2000;29:360-365

Murphy BJ, Laderoute KR, Chin RJ, Sutherland RM. Metallothionein IIA is upregulated by hypoxia in human A431 squamous cell carcinoma cells. *Cancer Res* 1994;54:5808-5810

Müller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH. P53 activates the CD95 (Apo-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* 1998;11:2033-2045

Nagase H, Woessner JF Jr. Matrix metallorpoteinases. *J Biol Chem* 1999;274:21491-21494

Nagata S, Golstein P. The Fas death factor. *Science* 1995;267:1449-1456

Nagata M, Fujita H, Ida H, Hoshina H, Inoue T, Seki Y, Ohnishi M, Ohyama T, Shingaki S, Kaji M, Saku T, Takagi R. Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. *Int J Cancer* 2003;106:683-689

Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001;7:683-694

Nakano M, Sogawa CA, Sogawa N, Mishima K, Yamachika E, Mizukawa N, Fukunaga J, Kawamoto T, Sawaki K, Sugahara T, Furuta H. Expression pattern of cisplatin-induced metallothionein isoforms in squamous cell carcinoma. *Anticancer Res* 2003;23:299-303

Nicholson DW, Thornberry NA. Caspases: killer proteases. *Trends Biochem Sci* 1997;22:299-306

Nitsch SM, Pries R, Wollenberg B. Head and neck cancer triggers increased IL-6 production of CD34+ stem cells from human cord blood. *In Vivo* 2007;21:493-498

Nix P, Cawkwell L, Patmore H, Greenman J, Stafford N. Bcl-2 expression predicts radiotherapy failure in laryngeal cancer. *Br J Cancer* 2005;92:2185-2189

Noguchi K, Tanimura H, Yamaue H, Iwahashi M, Tsunoda T, Tani M, Tamai M, Hotta T, Mizobata S, Arii K. Enhancement of tumor cell susceptibility to tumor-infiltrating lymphocytes by cisplatin. *J Cancer Res Clin Oncol* 1997;123:345-351

O-charoenrat P, Rhys-Evans P, Court WJ, Box GM, Eccles SA. Differential modulation of proliferation, matrix metalloproteinase expression and invasion of human head and neck squamous carcinoma cells by c-erbB ligands. *Clin Exp Metas* 1999:17:631-639

O-charoenrat P, Modjtahedi H, Rhys-Evans P, Court WJ, Box GM, Eccles S. Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. *Cancer Res* 2000;60:1121-1128

O-charoenrat P, Rhys-Evans PH, Eccles SA. Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* 2001;127:813-820

O-charoenrat P, Rhys-Evans P, Eccles S. A synthetic matrix metalloproteinase inhibitor prevents squamous carcinoma cell proliferation by interfering with epidermal growth factor receptor autocrine loops. *Int J Cancer* 2002;100:527-533

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000;288:1053-1058

Oh JW, Revel M, Chebath J. A soluble interleukin 6 receptor isolated from conditioned medium of human breast cancer cells is encoded by a differentially spliced mRNA. *Cytokine* 1996;8:401-409

Oh KJ, Lee SC, Choi HJ, Oh DY, Kim SC, Min do S, Kim JM, Lee KS, Han JS. Role of phospholipase D2 in anti-apoptotic signaling through increased expressions of Bcl-2 and Bcl-xL. *J Cell Biochem* 2007;101:1409-1422

Okabe M, Inagaki H, Murase T, Inoue M, Nagai N, Eimoto T. Prognostic significance of p27 and Ki-67 expression in mucoepidermoid carcinoma of the intraoral minor salivary gland. *Mod Pathol* 2001;14:1008-1014

Ondruschka C, Buhtz P, Motsch C, Freigang B, Schneider-Stock R, Roessner A, Boltze C. Prognostic value of MMP-2, -9, and TIMP-1,-2 immunoreactive protein at the invasive front in advanced head and neck squamous cell carcinomas. *Pathol Res Pract* 2002;198:509-515

Owen-Schaub LB, Radinsky R, Kruzel E, Berry K, Yonehara S. Anti-Fas on non-hematopoetic tumors: level of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. *Cancer Res* 1994;54:1580-1586

Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA,Deisseroth AB, Zhang WW, Kruzel E, et al. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol Cell Biol* 1995;15:3032-3040

Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;94:153-156

Partik G, Hochegger K, Schorkhuber M, Marian B. Inhibition of epidermal-growth-factor-receptor-dependent signalling by tyrphostins A25 and AG1478 blocks growth and induces apoptosis in colorectal tumor cells in vitro. *J Cancer Res Clin Oncol* 1999;125:379-388

Paulovich A, Toczyski D, Hartwell L. When checkpoints fail. *Cell* 1997;88:315-321

Pekkola-Heino K, Servomaa K, Kiuru A, Grénman R. Increased radiosensitivity is associated with p53 mutations in cell lines derived from oral cavity carcinoma. *Acta Otolaryngol* 1996;116: 341-344

Perez-Ordonez B, Beauchemin M, Jordan RC. Molecular biology of squamous cell carcinoma of the head and neck. *J Clin Pathol* 2006;59:445-453.

Pich A, Chiusa L, Navone R. Prognostic relevance of cell proliferation in head and neck tumors. *Ann Oncol* 2004;15:1319-1329

Pries R, Thiel A, Brocks C, Wollenberg B. Secretion of tumor-promoting and immune suppressive cytokines by cell lines of head and neck squamous cell carcinoma. *In Vivo* 2006;20:45-48

Popovic B, Jekic B, Novakovic I, Lukovic LJ, Tepavcevic Z, Jurisic V, Vukadinovic M, Milasin J. Bcl-2 expression in oral squamous cell carcinoma. *Ann N Y Acad Sci* 2007;1095:19-25

Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 2005;436:123-127

Rautajoki KJ, Marttila EM, Nyman TA, Lahesmaa R. Interleukin-4 inhibits caspase-3 by regulating several proteins in the Fas pathway during initial stages of human T helper 2 cell differentiation. *Mol Cell Proteomics* 2007;6:238-251

Riedel F, Götte K, Schwalb J, Bergler W, Hörmann K. Expresion of 92-kDa type IV collagenase correlates with angiogenic markers and poor survival in head and neck squamous cell carcinoma. *Int J Oncol* 2000;17:1099-1105

Riedel F, Zaiss I, Herzog D, Gotte K, Naim R, Hormann K. Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. *Anticancer Res* 2005;25:2761-2765

Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky DH, Gubler U, Sinigaglia F. Selective expression of an interleukin-12 receptor component by human T helper -1 cells. *J Exp Med* 1997;185:825-831

Rosenthal AL and Matrisian LM, Matrix metalloproteinases in the head and neck cancer. *Head Neck* 2006;28:639-648

Rothlein R, Czajkowski M, O´Neill MM, Marlin SD, Mainolfi E, Merluzzi VJ. Induction of intercellular adhesion molecule-1 on primary and continous cell lines by pro-inflammatory cytokines: regulation by pharmacologic agents and neutralizing antibodies. *J Immunol* 1988;141:1665-1669

Rubin Grandis J, Chakraborty A, Melhem MF, Zeng Q, Tweardy DJ. Inhibition of epidermal growth factor receptor gene expression and function decreases proliferation of head and neck squamous cell carcinoma but not normal mucosal epithelial cells. *Oncogene* 1997;15:409-416

Rubin JS, Day RM, Breckenridge D, Atabey N, Taylor WG, Stahl SJ, Wingfield PT, Kaufman JD, Schwall R, Bottaro DP. Dissociation of heparan sulfate and receptor binding domains of hepatocyte growth factor reveals that heparan sulfate-c-met interaction facilitates signaling. *J Biol Chem* 2001;276:32977-32983

Rudin CM, Kozloff M, Hoffman PC, Edelman MJ, Karnauskas R, Tomek R, Szeto L,Vokes EE. Phase I study of G3139, a bcl-2 antisense oligonucleotide, combined with carboplatin and etoposide in patients with small-cell lung cancer. *J Clin Oncol* 2004;22:1110-1117

Ruokolainen H, Paakko P, Turpeenniemi-Hujanen T. Serum matrix metalloproteinase-9 in head and neck squamous cell carcinoma is a prognostic marker. *Int J Cancer* 2005;116:422-427

Sakai E, Rikimaru K, Ueda M Matsumoto Y, Ishii N, Enomoto S, Yamamoto H,Tsuchida N. The p53 tumour-suppressor gene and ras oncogene mutations in oral squamous cell carcinoma. *Int J Cancer* 1992;52:867-872

Salgado R, Junius S, Benoy I, Van Dam P, Vermeulen P, Van Marck E, Huget P, Dirix LY. Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer* 2003;103:642-636

Salvesen GS, Dixit VM. Caspases: intracellular signalling by proteolysis. *Cell* 1997;91:443-446

Santini J, Formento JL, Francoual M, Milano G, Schneider M, Dassonville O, Demard F. Characterization, quantification, and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas. *Head Neck* 1991;13:132-139

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signalling pathways. *EMBO J* 1998;17:1675-1687

Scaffidi C, Schmitz I, Krammer PH, Peter ME. The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* 1999;274:1541-1548

Schendel SL, Azimov R, Pawlowski K, Godzic A, Kagan BL, Reed JC. Ion channel activity of the BH3 only Bcl-2 family member, Bid. *J Biol Chem* 1999;274:21932-21936

Schenk S, Hintermann E, Bilban M, Koshikawa N, Hojilla C, Khokha R, Quaranta V. Binding to EGF receptor of a laminin-5 EGF-like fragment liberated during MMP-dependent mammary gland involution. *J Cell Biol* 2003;161:197-209

Schlaefli E, Ehrke MJ, Mihich E.The effects of dichloro-trans-dihydroxy-bis-isopropyl-amine-platinum IV on theprimary cell-mediated cytotoxic response. *Immunopharmacology* 1983;6:107-122

Schoenermark M, Mitchell TI, Rutter JL, Reczek PR, Brinckerhoff CE. Retinoid-mediated suppression of tumour invasion and matrix metalloproteinase synthesis. *Ann N Y Acad Sci* 1999;30:466-486

Schwartz HS, Grindey GB. Adriamycin and daunorubucin: a comparison of antitumor activities and tissue uptake in mice following immunosuppression. *Cancer Res* 1973;33:1837-1844

Seiwert TY, Salama JK, Vokes EE. The chemoradiation paradigm in head and neck cancer. *Nat Clin Pract Oncol* 2007;4:156-171

Senderowicz AM. Novel small molecule cyclin-dependent kinases modulators in human clinical trials. *Cancer Biol Ther* 2003;2:S84-85

Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998;10:602-608

Sheikh MS, Carrier F, Johnson AC, Ogdon SE, Fornace AJ. Identification of an additional p53-responsive site in the human epidermal growth factor receptor promoter. *Oncogene* 1997;15:1095-1101

Sheu BC, Hsu SM, Ho HN, Lien HC, Huang SC, Lin RH. A novel role of metalloproteinase in cancer-mediated immunosuppression. *Cancer Res* 2001;61:237-242

Singh RK, Varney ML. IL-8 expression in malignant melanoma: implications in growth and metastasis. *Histol Histopathol* 2000;15:843-849

Skalova A, Leivo I, von Boguslawsky K, Saksela E. Cell proliferation correlates with prognosis in acinic cell carcinomas of salivary gland origin. Immunohistochemical study of 30 cases using the MIB-1 antibody in formalin-fixed paraffin sections. *J Pathol* 1994;173:13-21

Skibinski G, Skibinska A, James K. Hepatocyte growth factor (HGF) protects c-met-expressing Burkitt's lymphoma cell lines from apoptotic death induced by DNA damaging agents. *Eur J Cancer* 2001;37:1562-1569

Szlosarek PW, Balkwill FR. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol* 2003;4:565-573

Smalley KS, Brafford PA, Herlyn M. Selective evolutionary pressure from the tissue microenvironment drives tumor progression. *Semin Cancer Biol* 2005;15:451-459

Smilek P, Dusek L, Vesely K, Rottenberg J, Kostrica R. Correlation of expression of Ki-67, EGFR, c-erbB-2, MMP-9, p53, bcl-2, CD34 and cell cycle analysis with survival in head and neck squamous cell cancer. *J Exp Clin Cancer Res* 2006;25:549-555

Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994;76:959-962

Smith DJ, Jaggi M, Zhang W, Galich A, Du C, Sterrett SP, Smith LM, Balaji KC. Metallothioneins and resistance to cisplatin and radiation in prostate cancer. *Urology* 2006;67:1341-1347

Song JH, Song DK, Herlyn M, Petruk KC, Hao C. Cisplatin down-regulation of cellular Fas-associated death domain-like interleukin-1β-converting enzyme-like inhibitory proteins to restore tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human melanoma cells. *Clin Cancer Res* 2003;9:4255-4266

Sottrup-Jensen L, Birkedal-Hansen H. Human fibroblast collagenase-alpha-macroglobulin interactions. Localization of cleavage sites in the bait regions of five mammalian alpha-macroglobulins. *J Biol Chem* 1989;264:393-401

Speight PM, Barrett AW. Salivary gland tumors. *Oral Dis* 2002;8:229-240

Sporn MB, Robetrs AB, Goodman DS. The retinoids. Biology, chemistry and medicine 2nd ed. New York: Raven Press, 1994

Spreafico F, Vecchi A. The immunomodulatory activity of certain cancer chemotherapeutic agents: In: Mihich E, Sakurai Y, editors. Biological responses in cancer. Immunomodulation by anticancer drugs, vol 3. New York: Plenum, 1985, pp 131-154

Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. *Cancer Res* 2003;63:2948-2956

Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003;200:448-464

Stauber RH, Mann W, Knauer SK. Nuclear and cytoplasmic survivin: molecular mechanism, prognostic, and therapeutic potential. *Cancer Res* 2007;67:5999-6002

Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behaviour. *Ann Rev Cell Dev Biol* 2001;17:463-516

Stoll C, Baretton G, Ahrens C, Lohrs U. Prognostic significance of apoptosis and associated factors in oral squamous cell carcinoma. *Virchows Arch* 2000;436;102-108

Sunada F, Itabashi M, Ohkura H, Okumura T. p53 negativity, CDC25B positivity, and metallothionein negativity are predictors of a response of esophageal squamous cell carcinoma to chemoradiotherapy. *World J Gastroenterol* 2005;11:5696-5700

Surowiak P, Materna V, Maciejczyk A, Pudelko M, Markwitz E, Spaczynski M, Dietel M, Zabel M, Lage H. Nuclear metallothionein expression correlates with cisplatin resistance of ovarian cancer cells and poor clinical outcome. *Virchows Arch* 2007;450:279-285

Sutinen M, Kainulainen T, Hurskainen T, Vesterlund E, Alexander JP, Overall CM, Sorsa T, Salo T. Expression of matrix metalloproteinases (MMP-1 and MMP-2) and their inhibitors (TIMP-1, -2 and –3) in oral lichen planus, dysplasia, squamous cell carcinoma and lymph node metastasis. *Br J Cancer* 1998;77:2239-2245

Szlosarek PW, Balkwill FR. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol* 2003;4:565-573

Takaoka S, Iwase M, Uchida M, Yoshiba S, Kondo G, Watanabe H, Ohashi M, Nagumo M, Shintani S. Effect of combining epidermal growth factor receptor inhibitors and cisplatin on proliferation and apoptosis of oral squamous cell carcinoma cells. *Int J Oncol* 2007;30:1469-1476

Tashiro E, Tsuchiya A, Imoto M. Functions of cyclin D1 as an oncogene and regulation of cyclin D1 expression. *Cancer Sci* 2007;98:629-635

Taylor JL, Grossberg SE. The effects of interferon-alpha on the production and action of other cytokines. *Semin Oncol* 1998;25:23-29

Theocharis SE, Margeli AP, Klijanienko JT, Kouraklis GP. Metallothionein expression in human neoplasia. *Histopathology* 2004;45:103-118

Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. *Int J Exp Pathol* 2005;86:347-363

Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267:1456-1462

Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochimica et Biophysica Acta* 1985;827:36-44

Todd DG, Mikkelsen RB, Rorrer WK, Valerie K, Schmidt-Ullrich RK. Ionizing radiation stimulates existing signal transduction pathways involving the activation of epidermal growth factor receptor and ERBB-3, and changes of intracellular calcium in A431 human squamous carcinoma cells. *J Recept Signal Transduct Res* 1999;19:885-908

Tolcher AW, Chi K, Kuhn J, Gleave M, Patnaik A, Takimoto C, Schwartz G, Thompson I, Berg K, D'Aloisio S, Murray N, Frankel SR, Izbicka E, Rowinsky E. A phase II, pharmacokinetic, and biological correlative study of oblimersen sodium and docetaxel in patients with hormone-refractory prostate cancer. *Clin Cancer Res* 2005;11:3854-3861

Trauth BC, Klas C, Peters AM, Matzku S, Möller P, Falk W, Debatin K-M, Krammer PH. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 1989; 245:301-305

Trikha M, Corringham R, Klein B, Rossi JF. Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. *Clin Cancer Res* 2003;9:4653-4665

Trusolino L, Pugliese L, Comoglio PM. Interactions between scatter factors and their receptors: hints for therapeutic applications. *Faseb J* 1998;12:1267-1280

Tselepis C, Perry I, Dawson C, Hardy R, Darnton SJ, McConkey C, Stuart RC, Wright N, Harrison R, Jankowski JA. Tumour necrosis factor-alpha in Barrett's oesophagus: a potential novel mechanism of action. *Oncogene* 2002;21:6071-6081

Tsuchiya K, Shirato H, Nishioka T, Yamazaki A, Hashimoto S, Kagei K, Oomori K, Yasuda M, Shindo M, Miyasaka K. Pretreatment apoptotic scores do not predict response to radiation therapy in oropharyngeal squamous cell carcinoma. *Oral Oncol* 2001;37:159-163

Tsuji T, Noguchi M, Kido Y, Kubota H, Takemura K, Nakamori K, Hiratsuka H. Predictive assay of neoadjuvant chemotherapy in management of cancer. *Int J Oral Maxillofac Surg* 2007;36:15-19

Tsujimoto Y, Shimizu S. Bcl-2 family: life-or-death switch. *FEBS Lett* 2000;466:6-10

Uchida D, Kawamata H, Omotehara F, Nakashiro Ki, Kimura-Yanagawa T, Hino S, Begum NM, Hoque MO, Yoshida H, Sato M, Fujimori T. Role of HGF/c-met system in invasion and metastasis of oral squamous cell carcinoma cells in vitro and ist clinical significance. *Int J Cancer* 2001;93:489-496

Ugurel S, Rappl G, Tilgen W, Reinhold U. Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival. *J Clin Oncol* 2001;19:577-583

Uslu R, Jewett A, Bonavida B. Sensitization of human ovarian tumor cells by subtoxic CDDP to anti-Fas antibody-mediated cytotoxicity and apoptosis. *Gynecol Oncol* 1996;62:282-291

van Geelen CM, de Vries EG, Le TK, van Weeghel RP, de Jong S. Differential modulation of the TRAIL receptors and the CD95 receptor in colon carcinoma cell lines. *Br J Cancer* 2003;89:363-373

van Horssen R, Ten Hagen TL, Eggermont AM. TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. *Oncologist* 2006;11:397-408

Vaux DL, Cory S, Adams JM. Bcl2- gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1988;335:440-442

Vaux DL, Aguila HL, Weissman IL. Bcl-2 prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell mediated killing. *Int Immunol* 1992;4:821-824

Veneroni S, Silvestrini R, Costa A, Salvatori P, Faranda A, Molinari R. Biological indicators of survival in patients treated by surgery for squamous cell carcinoma of the oral cavity and oropharynx. *Oral Oncol* 1997;33:408-413

Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000;408:307-310

Vousden KH. p53 and PUMA: a deadly duo. *Science* 2005;309:1685-1686

Vousden KH. Outcomes of p53 activation – spoilt for choice. *J Cell Sci* 2006;119:5015-5020

Wang S, Coleman EJ, Pop LM, Brooks KJ, Vitetta ES, Niederkorn JY. Effect of an anti-CD54 (ICAM-1) monoclonal antibody (UV3) on the growth of human uveal melanoma cells transplanted heterotopically and orthotopically in SCID mice, *Int J Cancer* 2006;118:932–941.

Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;15:2922-2933

Wang X, DeFrances MC, Dai Y, Pediaditakis P, Johnson C, Bell A, Michalopoulos GK, Zarnegar R. A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. *Mol Cell* 2002;9:411-421

Watanabe H, Iwase M, Ohashi M, Nagumo M. Role of interleukin-8 secreted from human oral squamous cell carcinoma cell lines. *Oral Oncol* 2002;38:670-679

Wawryk SO, Novotny JR, Wicks IP, Wilkinson D, Maher D, Salvaris E, Welch K, Fecondo J, Boyd AW. The role of LFA-1/ICAM-1interaction in human leucocyte homing and adhesion. *Immunol Rev* 1989;108:135-161

Weichselbaum RR, Kufe DW, Hellman S, Rasmussen HS, King CR, Fischer PH, Mauceri HJ. Radiation-induced tumour necrosis factor-alpha expression: clinical application of transcriptional and physical targeting of gene therapy. *Lancet Oncol* 2002;3:665-671

Weinlich G, Eisendle K, Hassler E, Baltaci M, Fritsch PO, Zelger B. Metallothionein - overexpression as a highly significant prognostic factor in melanoma: a prospective study on 1270 patients. *Br J Cancer* 2006;94:835-841

Westermarck J, Kähäri VM. Regulation of matrix metalloproteinase expression in tumour invasion. *FASEB J* 1999,13:781-792

Whiteside TL. Immunobiology of head and neck cancer. *Cancer Met Rev* 2005;24:95-105

Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic interventions. *Semin Cancer Biol* 2006;16:3-15

Williams BA, Makrigiannis AP, Blay J, Hoskin DW. Treatment of the P815 murine mastocytoma with cisplatin or etoposide up-regulates cell-surface Fas (CD95) expression and increases sensitivity to anti-Fas antibody-mediated cytotoxicity and to lysis by anti-SD3-activated killer-T cells. *Int J Cancer* 1997;73:416-423

Williams LM, Gibbons DL, Gearing A, Maini RN, Feldmann M, Brennan FM. Paradoxical effects of a synthetic metalloproteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNF alpha processing in RA synovial membrane cell cultures. *J Clin Invest* 1996;97:2833-2841

Wilson GD, Saunders MI, Dische S, Richman PI, Daley FM, Bentzen SM. Bcl-2 expression in head and neck cancer: an enigmatic prognostic marker. *Int J Radiat Oncol Biol Phys* 2001;49:435-441

Wiman KG. Strategies for therapeutic targeting of the p53 pathway in cancer. *Cell Death Diff* 2006;13:921-926

Winograd-Katz SE, Levitzki A. Cisplatin induces PKB/Akt activation and p38(MAPK) phosphorylation of the EGF receptor. *Oncogene* 2006;25:7381-7390

Wu GS, Saftig P, Peters C, El-Deiry WS. Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity. *Oncogene* 1998;16:2177-2183

Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev* 2001;12:375-391

Xie X, Clausen OP, De Angelis P, Boysen M. The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue. *Cancer* 1999;86:913-920

Yan L, Anderson GM, DeWitte M, Nakada MT. Therapeutic potential of cytokine and chemokine antagonists in cancer therapy. *Eur J Cancer* 2006;42:793-802

Yang Z, Schumaker LM, Egorin MJ, Zuhowski EG, Guo Z, Cullen KJ. Cisplatin preferentially binds mitochondrial DNA and voltage-dependent anion channel protein in the mitochondrial membrane of head and neck squamous cell carcinoma: possible role in apoptosis. *Clin Cancer Res* 2006;12:5817-5825

Yang Z, Gagarin D, Ramezani A, Hawley RG, McCaffrey TA. Resistance to Fas-Induced apoptosis in cells from human atherosclerotic lesions: Elevated Bcl-X(L) Inhibits Apoptosis and Caspase Activation. *J Vasc Res* 2007;44:483-494

Yarden Y, Sliwkowski M. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127-137

Yin HF, Okada N, Takagi M. Apoptosis and apoptotic-related factors in mucoepidermoid carcinoma of the oral minor salivary glands. *Pathol Int* 2000;50:603-609

Yorioka CW, Coletta RD, Alves F, Nishimoto IN, Kowalski LP, Graner E. Matrix metalloproteinase-2 and -9 activities correlate with disease-free survival of oral squamous cell carcinoma patients. *Int J Oncol* 2002;20:189-194

Yoshizaki T, Maruyama Y, Sato H, Furukawa M. Expression of tissue inhibitor of matrix metalloproteinase-2 correlates with activation of matrix metalloproteinase-2 and predicts poor prognosis in tongue squamous cell carcinoma. *Int J Cancer* 2001;95:44-50

Yuen AP, Lam KY, Choy JT, Ho WK, Wong LY, Wei WI. Clinicopathologic significance of bcl-2 expression in the surgical treatment of oral tongue carcinoma. *Eur J Surg Oncol* 2002;28:667-672

Zarnegar J, Michalopoulos GK. The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *Cell Biol* 1995;129:1177-1180

Zhang W, Matrisian LM, Holmbeck K, Vick CC, Rosenthal EL. Fibroblast-derived MT1-MMP promotes tumor progression in vitro and in vivo. *BMC Cancer* 2006;6:52

Åkervall JA, Michalides RJ, Mineta H, Balm A, Borg A, Dictor MR, Jin Y, Loftus B, Mertens F, Wennerberg JP. Amplification of cyclin D1 gene in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin D1 overexpression. *Cancer* 1997;79:380-389

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