PROTEIN TYROSINE (DE-)PHOSPHORYLATION IN HEAD AND NECK CANCER

H.P. VERSCHUUR

STELLINGEN

behorend bij het proefschrift

PROTEIN TYROSINE (DE-)PHOSPHORYLATION IN HEAD AND NECK CANCER

in het openbaar te verdedigen
op dinsdag 17 mei 1994

door

HENDRIK PIETER VERSCHUUR

1. Zowel de cytosolaire als de membraan-gebonden proteïne tyrosine kinases en proteïne tyrosine phosphatases vertonen een verhoogde activiteit in hoofd-hals tumoren ten opzichte van normaal weefsel.

2. Enzymologisch gezien mag men de gezonde mucosa van patiënten, lijdend aan een hoofd-hals carcinoom, beschouwen als premaligne.

3. Membraan gebonden proteïne tyrosine kinase activiteit van tumor weefsel is gerelateerd met geslacht, lymfeklier metastasering, eerdere radiotherapie en lokalisatie. Cytosolair proteïne tyrosine kinase activiteit van tumor weefsel is gerelateerd met lymfeklier metastasering.

4. Tumor cellijnen afkomstig van plaveiselcel carcinomen van het hoofd-hals gebied worden door herbimycine A en tyrphostin 50875 geremd in hun groei. De mate van tyrosine phosphorylering wordt eveneens geremd door deze stoffen.

5. Het werken als research analist is niet voor alle arts-assistenten weggelegd maar de ervaring als zodanig is onontbeerlijk om de communicatie tussen klinische en fundamentele onderzoekers niet te laten verzanden in fundamentele tegenstellingen.

6. Dat het combineren van een promotie onderzoek met het volgen van een specialistische opleiding slecht is voor de sociale contacten tussen de promovendus en zijn omgeving is bekend. Dat het echter funest is voor het onderhouden van een zeker niveau van algemene ontwikkeling, blijkt vaak op een pijnlijke wijze tijdens de schaarse sociale ontmoetingen die de promovendus probeert te onderhouden met niet-medici.

7. Het al of niet voorschrijven van antibiotische prophylaxe bij chirurgische ingrepen is meestal gebaseerd op emotie en niet op ratio.

8. Het positief stimuleren van promovendi door woorden en daden heeft meer effect op het uiteindelijke resultaat dan het constant hameren op voortgang en deadlines.

9. De gedachte dat full-time werkende moeders slechte moeders zijn impliceert dat alleen Nederlandse moeders goed zijn.

10. Het vrijwel ontbreken van vrouwen in hogere posities is deels het gevolg van het feit dat mannen vrouwen niet alleen zien als collegae maar ook als toekomstige moeders en minnaressen.

11. Een arts heeft door zijn dagelijkse contacten met patiënten in het algemeen meer inzicht in de sociaal-maatschappelijk problemen van Nederland dan academici werkzaam in het bedrijfsleven of bij de overheid.

12. Volgens een Chinees spreekwoord moet een man in zijn leven niet alleen minstens een zoon maken, maar ook een boek schrijven en een boom planten. Dit laatste is, gezien de hoeveelheid kladpapier die voor het uitvoeren van de tweede opdracht nodig is, een ecologische noodzaak.

13. In een land van kruideniers regeren de boekhouders en niet de filosofen. De ontwikkeling van de gezondheidszorg in Nederland wordt daarom helaas bepaald door financiële doelstellingen en niet door ideeën.

14. De beeldenstorm was de laatste niet-calvinistische daad in Nederland.

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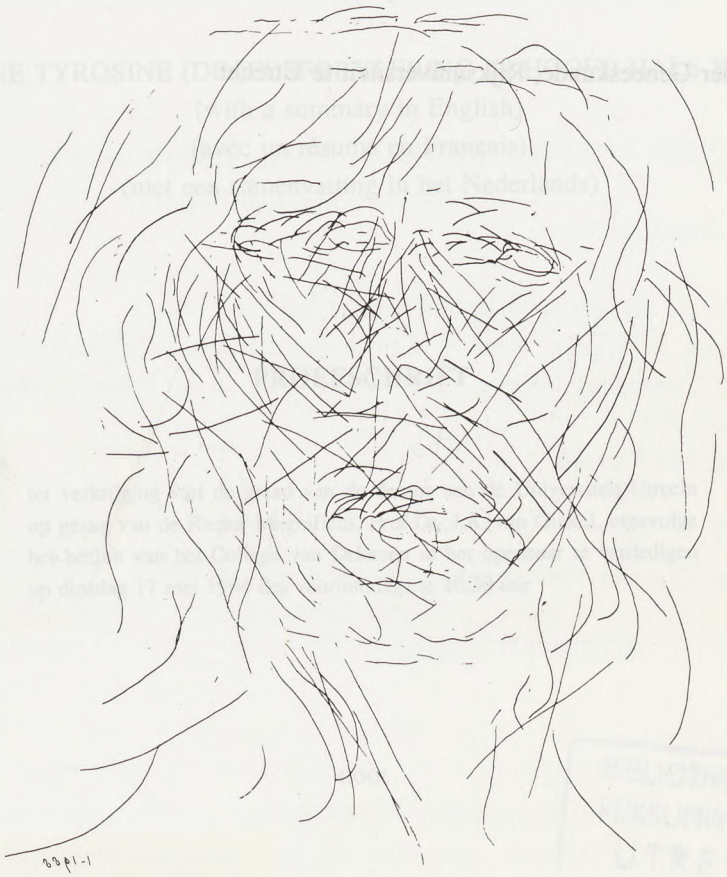

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PROTEIN TYROSINE (DE-)PHOSPHORYLATION IN HEAD AND NECK CANCER

PROTEINE TYROSINE (DE-)FOSFORYLERING IN HOOFD-HALS KANKER
(with a summary in English)
(avec un résumé en Francais)
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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CONTENTS

LIST OF ABBREVIATIONS

ATP	:	adenosine triphosphate
BSA	:	bovine serum albumin
CSK	:	*c-src* specific kinase
DTT	:	dithiothreitol
EDTA	:	ethylene diamine tetraacetic acid
EGF(r)	:	epidermal growth factor (receptor)
EGTA	:	ethylene glycol diamine tetraacetic acid
ELISA	:	enzyme linked immunosorbent assay
FGF(r)	:	fibroblast growth factor (receptor)
GAP	:	GTPase activating protein
HMA	:	herbimycin A
HNC	:	head and neck cancer
HNSCC	:	head and neck squamous cell carcinoma
HPV	:	human papilloma virus
IGF-1(r)	:	insulin-like growth factor-1 (receptor)
kD	:	kilodaltons
LAR	:	leucocyte common antigen related
P-ser	:	phosphoserine
P-thr	:	phosphothreonine
P-tyr	:	phosphotyrosine
PBS	:	phosphate buffered saline
PDGF(r)	:	platelet-derived growth factor (receptor)
PI3-kinase	:	phosphatidylinositol 3'-kinase
PLC-γ	:	phospholipase C-γ
PMSF	:	phenyl methyl sulphonyl fluoride
PGT	:	poly (glutamic acid;tyrosine 4:1)
PTK	:	protein tyrosine kinase
PTPase	:	protein tyrosine phosphatase
RNA	:	ribonucleic acid
SCC	:	squamous cell carcinoma
SCC-2	:	squamous cell carcinoma cell line (UMSCC-2)
SH2/3	:	*src* homology domain 2/3
T/N ratio	:	ratio of tumor versus non-tumor enzyme activities
TGFα	:	transforming growth factor α
TNM	:	tumor classification system (tumor nodes metastases)
tyr	:	tyrosine
UADT	:	upper aerodigestive tract
UICC	:	Union Internationale Contre le Cancer

CHAPTER 1

INTRODUCTION

LIST OF ABBREVIATIONS

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1.1 General aspects

Head and neck squamous cell carcinoma (HNSCC) accounts for approximately 5% of all human cancers. As these tumors originate in the mucosa of the upper aerodigestive tract (UADT), symptoms can already be present when the tumor is still in an early stage. Local invasion and regional lymph node involvement constitute the main cause of patients morbidity and mortality. However, distant metastasis and the occurrence of second primary tumors in the UADT and the lungs, important features of this disease, are difficult to anticipate and even more difficult to treat. Autopsy studies of patients who died of head and neck cancer (HNC) revealed clinical or occult distant metastases in as many as 50% of the cases. The occurrence of a second primary tumor in the lungs or the UADT appears to differ between the various studies. But one can assume that 5-35% of the patients treated for HNC will develop a second primary tumor there.

Five-year survival for the whole group is only ± 50% with a range of over 90% for small tumors at specific localizations and less than 30% for larger tumors with lymph node metastasis [11]. As in most other tumors, it is obvious that early detection is a prerequisite to improve cure rates and morbidity in this specific region.

Due to the localization of these tumors treatment can have an enormous impact on the patients. Radiotherapy, surgery, or a combination of both treatments, which are at present the preferred modalities, may result in serious functional and aesthetic impairment with unavoidable psychological consequences. Chemotherapy is currently useful only as palliative or adjuvant treatment. Despite recent advances in reconstruction after tumor resection and improved radiotherapy schemes, the overall cure rates of these tumors have hardly improved in the last decades. Nevertheless, these advances have certainly reduced the post-treatment morbidity.

Treatment results and cure rates are influenced by sex, localization, tumor size, presence of regional and distant metastasis, and histological characteristics. Individual prediction remains not very reliable for tumors with the same clinical and histological characteristics.

1.2 Molecular markers

Prediction and detection of recurrence, metastasis and second primary tumors remains difficult. Therefore it is mandatory to continue the search for molecular markers that can be used as prognostic parameters for head and neck squamous cell

carcinoma. These molecular markers can also provide a rationale for the development of non-surgical therapies like specific radio-labeled antibodies or new chemotherapeutic agents.
Analysis of these markers may result in several essential clinical applications.

I. A molecular marker could be used as a serum or tissue marker to diagnose and monitor patients with HNC.

II. If a molecular marker or a group of markers can predict the outcome of treatment of an individual patient or the occurrence of metastasis and/or second primary, irrespective of the already known clinical or histological parameters, we could change our treatment modalities into more or less aggressive ones, depending on the predicted prognosis.

III. If such a marker is at the extracellular surface, it could provide a target to direct specific antibodies labelled with cytotoxic or radioactive agents to localize or even destroy tumor masses [1].

IV. Markers can be the objective of basic research. If a molecular marker appears to be correlated to clinical and/or histological parameters, this finding can give us more insight into the process of oncogenesis. Perhaps this will lead us towards a future in which we can prevent, diagnose, and treat cancer in a more rational way.

1.3 Oncogenes and protein phosphorylation

Basic research on molecular markers in tumors has been focused on oncogenes and more recently on tumor suppressor genes. Many oncogenes appear to code for protein tyrosine kinases (PTKs), a group of enzymes which can phosphorylate proteins on tyrosyl residues.
The state of phosphorylation of tyrosyl residues in proteins plays a crucial role in regulating cell growth and differentiation [9,17-19,22]. The steady state level of tyrosine phosphorylation of cellular proteins depends on the balance of the action of the PTKs and the protein tyrosine phosphatases (PTPases) [9,18].
Alteration in the regulation of the phosphorylation state of tyrosyl residues on various cellular proteins appears to be an important pathway towards neoplastic

transformation [2,5]. This alteration may arise through either deregulation or changed expression of PTKs or PTPases.
Amplification or expression of mutated forms of oncogenes with PTK activity has been directly associated with neoplastic growth [6,12] and is sometimes related to the clinical behavior of certain tumors and thus to the prognosis of the patient [6,8,13,16,23].

The relationship between PTK activity and the growth and/or differentiation of human tumors has been described by several authors [3,4,7,10,14,15,20,21]. Much less work has been done on the role of PTPases in growth and differentiation of human tumors. Further characterization of the role and regulation of PTK and PTPase activity in tyrosyl phosphorylation in HNC may provide clues for understanding the complex process of signal transduction during cell growth and differentiation of both normal and neoplastic cells. Furthermore this may provide the new molecular markers so urgently needed in clinical practice.

1.4 Objectives of this study

As we consider that the state of phosphorylation of tyrosyl residues in proteins plays a crucial role in regulating cell growth and differentiation, we have addressed several questions in this thesis regarding the enzymes involved in the tyrosine phosphorylation.

I. Are there differences between the PTK activity in tumor tissue compared to normal tissue?

II. Are there differences between the PTPase activity in tumor tissue compared to normal tissue?

III. Are there differences in the level of phosphorylated tyrosine residues in tumor tissues compared to normal tissues?

IV. Can PTPase activity be detected with enzyme histochemical techniques and can differences in PTPase activity be localized?

V. Is the PTK and PTPase activity in tumor tissue and non-tumor tissue correlated to any clinical and/or histological parameter?

VI. Can the determination of the PTK and PTPase activity in tumor tissue and non-tumor tissue be used as an independent prognostic factor for disease-free intervals or occurrence of a second primary tumor?

VII. Can the specific inhibition of PTK activity in cultured tumor cells give more insight into the signaling pathways of malignant transformation?

1.5 Outline of this thesis

In Chapter 2 we discuss the most important aspects of oncogenesis, protein (de-)phosphorylation and signal transduction in malignancy. We also review the research on oncogenes and tumor suppressor genes in HNC. Finally, we also briefly indicate some other molecular markers, even if they are not (yet) linked to oncogenes, tumor suppressor genes or protein phosphorylation.

Chapter 3 presents the results of a pilot study concerning the determination of PTK activity in laryngeal cancer.

Chapter 4 describes a pilot study on PTK and PTPase activity in HNC. A study concerning the level of phosphorylation of tyrosyl residues in HNC is also reported.

Chapter 5 deals with the enzyme histochemical detection and localization of PTPase activity in head and neck cancer.

In Chapter 6 the clinical relevance of the determination of PTK and PTPase activity in head and neck cancer is reported in a large series of patients. Correlations with known clinical and/or histological parameters are described. Its value as an independent prognostic factor for the disease-free interval and for the development of second primaries is also discussed.

Chapter 7 presents the first results of cell growth inhibition studies in cultured tumor cells with specific PTK inhibitors. The effect of the inhibitors on phosphotyrosine levels and PTK activity is also described.

The interpretation of the results described in these chapters is discussed in Chapter 8. Additionally, some suggestions are made for future studies concerning tyrosine (de-)phosphorylation in head and neck squamous cell carcinomas.
Finally, a summary of this thesis is given in respectively English, French, and Dutch.

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CHAPTER 2

ONCOGENES AND SIGNAL TRANSDUCTION IN HEAD AND NECK CANCER

Hendrik P. Verschuur, Gert Rijksen, Gerard E.J. Staal, and Gert Jan Hordijk

(submitted for publication)

CHAPTER 2

ONCOGENES AND SIGNAL TRANSDUCTION IN HEAD AND NECK CANCER

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2.1 Oncogenesis

Oncogenesis is considered to be a complex multistep process of which only particular fragments have been elucidated. Several steps are suggested, each of which limits the rate of the process [23,133]. Experimental models have defined at least three distinct steps: initiation, promotion, and progression [41,43]. These steps can be recognized in human colon adenocarcinoma when normal mucosa changes via benign polyps into malignant carcinoma and where molecular changes can be identified at each step [23].

Initiation is the first requirement for oncogenesis. Animal model systems have shown that a single chemical agent or a certain dose of radiation can induce a point mutation which can change the expression and/or the activity of certain genes. Sometimes an additional tumor promoter is needed to promote a further malignant transformation. Inversion of the sequence of tumor initiator and promoter does not induce a malignant change.

In human cancer, anecdotal reports support the theory of induction of oncogenesis by chemical agents or radiation. For instance, at the beginning of this century, scrotal cancer in chimney sweepers was associated with aromatic benzene exposition coming from the chimney soot. More recently, persons who received large doses of radiation developed certain forms of leukemia. Precise examples of tumor-promoting agents in human cancer are not known. However, in grade III dysplasia of the laryngeal mucosa, epidemiological studies have shown that tobacco smoke does increase the immediate risk of degeneration into a squamous cell carcinoma [56]. This is seen less often when patients stop smoking. In this example, tobacco smoke could be a promoting agent in oncogenesis. Yet experimental models have thusfar not been able to confirm these findings.

The most widely accepted paradigm of oncogenesis is based on the interaction between genetic predisposition and environmental factors. After a multistep process, the balance between positive and negative growth regulatory mechanisms is disrupted, and the normal cell is transformed into a cancer cell.

Genetic predisposition for cancer can be seen in several types of tumors where relatives have a higher risk of developing the same malignancy. Several environmental factors have been demonstrated or suspected to be directly or indirectly linked to certain types of tumors. Ultraviolet light, viruses, and certain chemicals can cause tumors in experimental models. And the consumption of

alcohol and tobacco is clearly linked with cancer of the lung and the upper aerodigestive tract (UADT).
The concept of predisposition is derived from the changes seen in certain genes which are responsible for the normal growth and differentiation. These are called proto-oncogenes and tumor suppressor genes.

2.2 Proto-oncogenes and oncogenes

In 1911, Rous discovered that a cell-free extract from chicken sarcoma could form sarcomas when injected in animals, but he did not yet realize that the oncogenetic potential was transmitted by a piece of viral RNA. Later others discovered that it was an RNA retrovirus, which was incorporated after a reverse transcription into the host DNA, that was responsible for the oncogenesis of the sarcoma. The name viral oncogene ('v-onc') was then launched. Many other viral oncogenes have since been identified. Very few of them are thought to be responsible for human oncogenesis. But at about the same time, it was found that certain human genes had great similarities with viral oncogenes. Much later it became clear that all human cells possess these genes, which are known as proto-oncogenes (also called '*c-onc*'). Bishop and Varmus received the Nobel Prize in 1989 for their work in which they demonstrated that the viral oncogenes are derived from their cellular counterparts, the proto-oncogenes.
Furthermore, for several proto-oncogenes homologous sequences have been discovered in all organisms studied, from flies to humans, with a high degree of conservation across their evolution [110]. The proto-oncogenes regulate the proliferation and differentiation of normal cells. If activated into oncogenes, they may be associated with the process of deregulated proliferation and differentiation of tumor cells. Proto-oncogenes can be activated into oncogenes by a minor change, such as the substitution of a nucleotide in the DNA. Other mechanisms of activation include insertion of a promoter or enhancer, deletion of an inhibiting gene, translocation of a part of the chromosome, or amplification. This activation may lead to an increase in the oncogene products or to the production of altered gene products, both resulting in a malignant transformation of the cell.

The above-mentioned environmental factors probably play a role in oncogenesis during or through the conversion of proto-oncogenes into active oncogenes.

The hypothesis that activation of multiple oncogenes (perhaps partly by environmental factors) is necessary for the development of cancer has proven to be particularly powerful in explaining oncogenesis at the molecular level.

This hypothesis is supported by several findings. An initial connection was made through the study of the *ras* and *myc* oncogenes. Each of the oncogenes alone was unable to transform rat embryo fibroblasts on its own. But the two, cooperating together, elicited a fully tumorigenic phenotype [69].

Table 1. A classification of oncogenes by the function or localization of their products.

Protein tyrosine kinases	*erb-B/neu*/HER2 *fms* *src* *fes* *abl* *ros*
Serine/threonine protein kinases	*raf* *mos*
Growth factors	*sis* *int-2* *hst-1*
GTP-binding proteins	H-*ras* K-*ras* N-*ras*
Adaptor molecules	*shc* *grb2/sem-5*
Nuclear proteins	c-*myc* N-*myc* L-*myc* *myb* *fos* *jun* *ski*
Miscellaneous	*prad-1/ems-1* *bcl*

In this experimental model two oncogene activations (or steps) are needed for full transformation. In some models only one activated oncogene is needed for full transformation, but these are mostly experiments with immortalized cell types like the NIH3T3 cells [94]. This immortalization can be rationalized as a premalignant phenotype. Because it resembles that induced by an activated oncogene, this phenotype is responsive to transformation by a single oncogene. Thus, in the human 'model', several steps of activated oncogenes are required for cancer to develop.

Oncogenes can be classified either according to the function of their products, the oncoproteins (e.g. PTK activity) or to their localization in the cell (eg. membrane-bound). A classification is shown in Table 1. The group of oncogene products with protein kinase activity can be divided into protein tyrosine kinases and serine/threonine kinases. Members of both groups can be membrane-bound, cytoplasmic, or nuclear. The oncogene products can be found in the extracellular space, as (transmembrane) receptors, as membrane-associated or cytosolic proteins or as intranuclear proteins.

Increasing evidence suggests that the signal transduction from growth factor receptor to nucleus and from nucleus to proliferation is regulated by a complex equilibrium between protein tyrosine phosphorylation and dephosphorylation [37,119].

2.3 Tumor suppressor genes

Another class of genes involved in oncogenesis comprises the group of tumor suppressor genes which were discovered by studying hereditary cancers like juvenile retinoblastoma [65]. These genes are considered to be responsible for at least one step of oncogenesis following their inactivation or aberrant expression. Tumor suppressor genes are believed to down-regulate inapt proliferation and are therefore the vanguards of the normal cell cycle. Inactivation or aberrant expression of the genes on both alleles can undermine their regulating function and thus induce or promote malignant transformation.
By now there is a large body of evidence showing that qualitative and quantitative changes in oncogenes are involved in oncogenesis. At the same time, the importance of the role of tumor suppressor genes seems to be gaining recognition.

The presence of oncogenes and tumor suppressor genes and the overexpression or inactivation of their products can be examined in human tumors at DNA level, RNA

level, protein level, or with respect to protein function. Both quantitative biochemical assays and localizing histochemical techniques can be used.

Several other molecular markers have been investigated which cannot be assigned a place (yet) in the framework of oncogenes and tumor suppressor genes. Some markers are listed in Table 2. The terms 'oncogenes' and 'tumor suppressor genes' are still not clearly defined. The name suggests that they are genes encoding for proteins responsible for the induction, progression, or inhibition of malignant transformation. It is probably a semantic discussion whether all molecular changes observed during oncogenesis may be called oncogenes or tumor suppressor genes or whether they have to be designated as 'markers' until an encoding gene has been identified. In the meantime, these markers represent a miscellaneous group of molecular changes seen in the cytoplasm, nuclear membrane, cell membrane or even in the serum of the patient.

2.4 Signal transduction by protein tyrosine (de-)phosphorylation

Protein phosphorylation and dephosphorylation is considered to be an important mechanism in the regulation of cell proliferation and differentiation [37,119]. Protein tyrosine phosphorylation is a result of the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). The former (PTK) specifically catalyze the transfer of a phosphate group from ATP to a tyrosine residue of the substrate protein. The latter (PTPase) remove the phosphate group and return the substrate to its unphosphorylated state. When Hunter and Sefton discovered that the oncogene *src* possessed PTK activity, an opening was made to explore the role of oncogenes in signal transduction. Later it became known that many oncogene products (oncoproteins) belong to the group of the protein tyrosine kinases or interfere in tyrosine phosphorylation. As oncogenes play a crucial role in the development of tumors, tyrosine phosphorylation is probably the 'messenger' of the oncogenic signal transduction. By understanding the mechanisms of tyrosine phosphorylation, we may get closer to elucidating growth control in tumor cells.

Recently several protein tyrosine phosphatases have been characterized (reviewed in [119]). It is known that many protein tyrosine kinases are products of oncogenes and protein tyrosine phosphatases counteracts the effect of PTKs. Accordingly, one may consider the encoding genes of PTPases as tumor-suppressor genes [37,78].

Protein tyrosine kinases can be divided into two categories: (membrane-bound) receptor PTK, and non-receptor PTK. Most of the receptor PTK are growth factor receptors. These large proteins have an extracellular ligand-binding domain, a short (hydrophobic) transmembrane region, and an intracellular catalytic domain. Binding of a ligand to the extracellular ligand-binding domain results in conformational change of the receptor and in an activation of the intracellular PTK catalytic domain. Autophosphorylation of the intracellular domain has been suggested to precede the activation of the growth factor receptors. Autophosphorylation also induces their association with several target proteins, which bind to specific tyrosine phosphorylated sites within noncatalytic regions of the receptors. These receptor-binding proteins, such as adaptor molecules like Shc and Grb2, are able to recruit other signal transduction components [98]. Other proteins, which include phospholipase C-γ (PLC-γ), phosphatidylinositol 3'-kinase (PI3-kinase), Ras GTPase-activating protein, and *src*-family tyrosine kinases, are themselves regulators of intracellular signaling pathways. Hence they are logical receptor targets [24,77,91]. Epidermal growth factor receptor (EGFr), insulin receptor, insulin-like growth factor-1 (IGF-1) receptor, platelet-derived growth factor (PDGF) receptor, and fibroblast growth factor (FGF) receptor are all examples of membrane-bound receptor protein tyrosine kinases.

It is very likely that activated growth factor receptors take part in the signal transduction process which at the end produces a factor identical or similar to the original growth factor [122]. After secernation, these factors can restimulate the receptor, thereby creating an autocrine loop.
An example of this loop is the production by certain tumor cells of a 'transforming growth factor α' (TGFα) which acts as a ligand for the EGF receptor. The EGFr is known to have an increased expression in human squamous cell carcinomas [5,9-11,15,60,63,82,95,100,104,111,139]. Stimulation by TGFα could result in an enhanced proliferation of these SCC as seen in tumor cell lines after stimulation by EGF. Therefore, stimulation could lead to an increase in TGFα or even EGFr production [14,88,118].
Oncogenes can also code for membrane-bound receptors very similar to growth factor receptors. The *c-erbB2* (also called *neu* or Her2) oncogene, for example, codes for a receptor-like protein which is very similar to the EGFr. The viral counterpart of the proto-oncogene *c-erbB1* (encoding the EGF receptor) is *v-erbB*, which codes for a truncated form of the EGFr lacking the extracellular ligand-binding domain. Due to its absence, the intracellular PTK domain is probably

constitutively activated. Another example of oncogene products resembling growth factor receptors is *c-fms* for CSF1-receptor.

The best-known example of non-receptor PTKs is formed by the oncogenes of the *src*-family. Apart from the PTK domain, each family member contains SH2 and SH3 (src homology) domains, which are responsible for the recognition of target proteins. The SH2 and SH3 domains are important because several proteins involved in signal transduction, like (PLC-γ), phosphatidylinositol 3'-kinase (PI3-kinase), and Ras GTPase-activating protein, have been shown to possess these domains [7,24,77,91,98]. These domains are also involved in the association of those proteins with activated growth factor receptors.

This chapter gives an overview of the most important oncogenes and tumor suppressor genes and their products that have been investigated in head and neck cancer. Some oncogenes, that have not been observed in head and neck cancer yet are also discussed if a role in oncogenesis of HNC is probable.
The speed at which research on oncogenes and tumor suppressor genes in HNSCC is being published prohibits a complete, up-to-date overview of this subject. Nevertheless, as the amount of work already published is so vast and confusing, we believe that an overview, though incomplete, could not only help the clinician but also the basic researcher to see some order in the apparent chaos of data. We also briefly indicate some other molecular markers, even if they do not fit into the present definition of oncogenes and tumor suppressor genes.

2.5 The transmembrane receptors

2.5.1 Epidermal growth factor receptor (EGFr)

Many oncoproteins are transmembrane receptors acting as signal transducers between the extracellular and the intracellular space. Many are growth factor receptors with an intracellular protein tyrosine kinase activity [122]. The intracellular targets of these growth factor receptors have not been identified yet in HNSCC, but some candidates have been identified in hematological neoplasms and tumor cell lines [106,122].
The best-known example of this group is the EGF receptor, which has been extensively investigated in cancer of the upper aerodigestive tract [5,9-11,15,60,63,82,95,100,104,111,139]. It is a 170 kD transmembrane protein with an extracellular ligand-binding domain, a short transmembrane domain, and an

intracellular part which has PTK activity. The 'normal' ligand for EGFr is EGF, but it has been shown that transforming growth factor alpha (TGFα) also binds to and activates the receptor [137].
In head and neck cancer, there are no reports on EGF production by tumor cells. No EGF expression has been found in normal epithelial tissues either [137]. The impact of exogenous EGF on in vitro experiments in HNC is not very clear [131,132]. In contrast, TGFα has been found in both normal epithelial cells and squamous cell carcinomas [15,88,118,138]. Interestingly, tumor associated eosinophils, which are known to correlate with metastasis and prognosis, are the major source of TGFα in HNC [117]. TGFα expression also correlates with cell proliferation when hamster cheek pouches are chemically induced to develop oral cancer with DMBA [138]. However, TGFα alone is not enough for malignant transformation. EGFr overexpression is also needed, as has been shown in transgenic mice [16]. Therefore, it is likely that perturbations in expression of both TGFα and EGFr can be important in malignant transformation of epithelial tissues. An overexpression of TGFα and EGFr in the same cell line or tumor is highly suggestive of an autocrine or paracrine mechanism of growth [118,137,138].

EGFr amplification and overexpression has also been reported in various other tumor sites, including mamma, vulva, bladder, thyroid, esophagus and lung. In vitro experiments have shown that EGFr overexpression alone is not enough for transformation of fibroblasts; the cells require the addition of EGF to transform [94]. The overexpression of EGF receptor in head and neck cancer has been reported numerous times [5,9,15,60,62,72,82,83], but conflicting reports on EGFr gene amplification exist. Gene amplification was reported to be either absent or present in up to 30% of all patients [21,58,60,72,80,95,113]. Immunohistochemical detection of EGFr in normal mucosa showed almost exclusive staining of the basal cells, suggesting a role for this receptor in proliferating cells. Most of the cells in dysplasia or carcinoma regions stained positively, especially on the invasive margins [62,82]. Furthermore, the staining intensity correlated with the degree of differentiation [9,89]. Interestingly, in one report EGFr overexpression was also found in the non-tumorous mucosa of the cancer patients as well as normal mucosa of persons with heavy drinking and smoking habits [5].
By immunohistochemical detection of EGFr, a differentiation could be made between mild to moderate dysplasia of the larynx versus severe dysplasia and carcinoma in situ [83].
Another transmembrane oncogene is *c-erbB2* (also called *neu* or Her2), which shares considerable homology with *c-erbB1*, the coding proto-oncogene for EGFr. It also

has an intracellular tyrosine kinase domain and has been found amplified and/or overexpressed in malignancies of the breast [96] and pancreas [49]. In breast cancer, *c-erbB2* amplification is related to disease-free interval and can be considered an independent prognostic factor [112].
Amplification of *c-erbB2* in head and neck cancer has also been reported [63,67,72,80,113]. In one report, overexpression of *c-erbB2* was found in 60% of the patients by applying immunohistochemical techniques. Remarkably, the oncogene product was not detected on the membrane but in the cytoplasm. Cytosolic staining could also be observed in breast cancer [112].
A gradual increase of staining intensity, from mild dysplasia to carcinoma, with an immunoperoxidase assay using a monoclonal antibody against *c-erbB2* has been reported [57]. The significance of this finding is not clear yet, but it has also been noticed in breast cancer.

2.6 Membrane-associated oncoproteins

These are mostly proteins involved in the intracellular signal transduction but who are in one way or another, attached to the cytosolic part of the membrane.

2.6.1 *Ras*-family

This family consists of three functional genes: H-*ras*, K-*ras* and N-*ras*. These encode highly similar proteins with molecular weights of 21,000. The *ras* oncoprotein p21 is homologous to G-proteins, which are membrane-associated GTPase enzymes, except that it needs a co-protein GAP (GTPase-activating protein) to exert its activity. *Ras* proteins play an important role in signal transduction. Recent reports suggest that the activation of *ras* could be triggered by ligand-bound cell surface receptors that possess PTK activity (like EGFr). Several adaptor molecules and other factors are supposed to mediate this activation [7,24,98]. *Ras* is supposed to transmit the signal further downstream via a series of cytosolic serine/threonine protein kinases [7,73,77].

In chemical oncogenesis, specific base mutations of codon 12 and 61 of the *ras* gene has been seen after induction with NMU (N-nitrose-N'-methylurea), respectively DMBA. These mutations lead to a prolonged activated state of p21 ras. *ras* mutations have been found in many solid tumors, including colon, salivary gland, and thyroid cancer. High percentages of *ras* mutations have also been found in premalignant adenomatous polyps of the colon as well as in thyroid adenomas.

These clinical findings, as well as findings with chemically induced tumors, suggest an early role for *ras* in oncogenesis. In breast cancer, a correlation was found between high p21 ras expression and poor prognosis [130]. In contrast, other reports described a favorable prognosis in neuroblastomas expressing high levels of p21 ras. In non-small cell lung cancer, the presence of *ras* mutations was associated with a short disease-free interval [81]. These conflicting reports make it difficult to evaluate the precise role of *ras* in oncogenesis.

The reports on *ras* amplification, mutation, and/or overexpression in head and neck cancer are also confusing. A *ras* mutation could be detected in less than 5% of the patients, and no DNA amplification was reported [40,52,80,99,109,114,135]. Reports coming from India, present a different picture. There H-*ras* was found to be mutated in 35% of the oral carcinomas [101], while K- and N-*ras* were amplified (together with c-*myc* or N-*myc*) in 17%, respectively 30% of the cases [102]. The authors explain these high percentages by pointing out the different risk factors found in India, such as betel quid chewing, which is not common outside the subcontinent. While *ras* mutations or gene amplifications apparently do not play an important role in head and neck cancer outside India, several reports indicate that increased p21 ras expression is important in head and neck oncogenesis [35,53,103]. Even a correlation between high p21 ras expression and poor prognosis was found [35]]. Another report showed a relation between p21 expression and smoking habits in Japan, which was not found in a British series [28]. However, in the latter series a relation between smoking and simultaneous overexpression of p21 and a mutated form of the tumor suppressor gene p53 (see Section 2.9.2) was found. Overexpression of H-*ras* en K-*ras* has also been reported [28] even in the absence of genetic changes in the H-*ras* gene [109].

Tentatively, we may conclude that genetic mutations in the *ras* family may be important in an early stage of oral cancer related to betel quid chewing, while H-*ras*, K-*ras*, and p21 ras overexpression could be important in a later phase of oncogenesis in tobacco-related head and neck cancer.

2.6.2 *Src* family

This family consists of at least eight genes that share a considerable amount of homology. Of these eight, *src* is the best known. Some of them, like *fgr*, *lck*, *hck* and *blk*, are only expressed in hematopoietic cells. Others, like *src*, *yes*, *lyn* and *fyn*, are ubiquitously expressed. The *src* gene product is a 60 kD phosphoprotein

($pp60^{c\text{-}src}$) and is attached to the intracellular part of the cell membrane by myristic acid.

Apart from the PTK domain, $pp60^{c\text{-}src}$ also contains the SH2 and SH3 (src homology) domains, which are responsible for the recognition of target proteins. The SH2 and SH3 domains are important because several proteins involved in signal transduction, like PLC-γ, PI3-kinase, and Ras GTPase-activating protein, possess these domains [7,24,77,91,98]. These domains are also involved in the association of those proteins with activated growth factor receptors.

Activated PGDF receptors, which show PTK activity, interact with $pp60^{c\text{-}src}$ through the SH2 domain. Complex formation is accompanied by enhancement of the $pp60^{c\text{-}src}$ PTK activity, with subsequent translocation of $pp60^{c\text{-}src}$ from the plasma membrane to the cytosol [127].

Interestingly, in cells stimulated with PGDF, $pp60^{c\text{-}src}$ is phosphorylated at tyrosine as well as at serine residues. It is not clear whether these phosphorylation events are necessary for its increased PTK activity or whether they play a role in signal transduction.

On the other hand, $pp60^{c\text{-}src}$ can be activated by dephosphorylation of $tyrosine^{530}$. Recently *c-src* kinase (CSK) has been identified as a tyrosine kinase able to phosphorylate $pp60^{c\text{-}src}$. When increasing the amount of CSK in an in vitro experiment, the level of phosphorylation of tyr^{530} increased. At the same time, a decrease of $pp60^{c\text{-}src}$ PTK activity was observed [84,141]. The exact mechanism of phosphorylation and dephosphorylation of *src*-like products has not been completely elucidated yet.

Increased $pp60^{c\text{-}src}$ protein expression has been found in several tumors, including colon carcinoma, skin cancer, and sarcomas. In breast cancer, 70% of the increased cytosolic PTK activity found in these tumors originates from the $pp60^{c\text{-}src}$ protein. This was not only caused by an increased expression of the protein but also by an increased specific activity of the enzyme [87]. Unpublished data from our laboratory (see Chapter 7) suggest that $pp60^{c\text{-}src}$ may play a comparable role in the total PTK activity in head and neck cancer.

2.7 Nuclear oncoproteins

2.7.1 *Myc* family

The *myc* family has been studied extensively in most human malignancies. It consists of three genes: c-*myc*, N-*myc* and L-*myc*; all three code for a 62 kD protein.

These proteins appear to be involved in normal cell proliferation and differentiation. Their exact function has not been elucidated, but it is likely that they act as transcriptional regulatory proteins [22]. In several human tumors gene rearrangements or overexpression of gene products have been reported [22,31]. In head and neck cancer, both amplification and overexpression of *myc* have been reported [30,34,50,72,80,97,102]. Although no correlation with any clinicopathological parameters was found, there appeared to be an independent correlation with prognosis. An interesting aspect in *myc* overexpression is that it is accompanied most of the time with *ras* overexpression [29,31,102,115]. This simultanous expression is elegantly confirmed in vitro. Embryo fibroblasts are transformed only when *myc* and *ras* are both transfected into the cell [69]. Co-amplification of *myc* and the oncogene *int-2* has also been reported [72,80], but as *int-2* is located on the important genetic region 11q13, together with other oncogenes (see further below), it is difficult to evaluate these findings. The same co-expression is observed with *myc* and EGFr overexpression; the latter is known to happen frequently in HNC (see also Section 2.5.1.) [72,115,140].

2.8 Oncogenes located on the 11q13 region (*int-2*, *bcl-1*, *hst-1* and *prad-1/ems-1*)

Another group of oncogenes that are usually classified together, due to their localization on the same chromosome, is the group consisting *int-2*, *bcl-1*, *hst-1* and *prad-1/ems-1*. These oncogenes are all located on the long arm of chromosome 11, in region 11q13. All these genes are located within a 250 kb region. It is therefore difficult to detect amplification of separated genes. It is even possible that *prad-1/ems-1* is the same as *hst-1*, as they lie within 120 kb of each other [68]. The *int-2* and *hst-1* genes encode for members of the fibroblast growth factor family [28]. The fibroblast growth factors function in angiogenesis and as mutagenic growth factors. *Prad-1/ems-1* is believed to be a member of the cyclin family. The cyclins are important during the cell cyclus and are overexpressed during increased cell proliferation [68]. Amplifications of the 11q13 region have been found in esophagal carcinoma [8], breast cancer [68], and head and neck cancer.

Amplifications of the oncogenes located in the 11q13 region in HNC have been reported in 8% to 52% of all patients [4,72,80,113,142]. At present, amplification of these genes has not been related to clinicopathological parameters or clinical outcome.

2.9 Tumor suppressor genes

Another major class of genes involved in oncogenesis is the group of tumor suppressor genes. These genes are now supposed to play an important role in the prevention of malignant transformation. Tumor suppressor genes are believed to act as down-regulators of inapt proliferation. The confirmation of their existence has been shown only recently. Oncogenes act as dominant genes, while tumor suppressor genes can be considered as recessive genes. Consequently, to demonstrate that a loss of function of a tumor suppressor gene can cause a tumor, both copies of that gene have to be malfunctioning instead of one, as in the case of an oncogene.

2.9.1 Rb gene

The first tumor suppressor gene described is associated with retinoblastoma: the Rb-gene. Knudson [65] hypothesized in 1971 that the difference in juvenile (inherited) retinoblastoma and the sporadic (non-inherited) retinoblastoma was due to the fact that in the inherited form, children already miss an allele due to a germ-line mutation. When the second allele malfunctions because of a somatic mutation, a tumor will grow. In the non-inherited form, both alleles must malfunction in the same cell in order to create a tumor; of course, this is much less likely [65]. While loss of both alleles may lead to malignant transformation, replacement of a normal Rb allele in a tumor cell can cause it to revert to a normal state, as has been shown in vitro.

The exact function of the Rb product $p105^{Rb}$ is unknown but probably very complex. It appears that the dephosphorylated state of $p105^{Rb}$ is the active form, while phosphorylation at several serine/threonine residues by cdc 2 kinase deactivates this protein.

To date, only one anecdotal report has been published linking a case of sinonasal carcinoma with Rb loss of heterozygosity (LOH).

An interesting aspect of head en neck cancer are the differences found in $p105^{Rb}$ binding affinities of certain human papilloma virus (HPV) oncoproteins [19]. Human papilloma viruses, especially subtypes 6 and 16, are known to be associated with benign and malignant head and neck tumors [44,59,61,108]. The E7 protein of HPV16 has a stronger binding affinity to $p105^{Rb}$ than the same protein of HPV6. Therefore, the HPV16 is more likely to block the activity of $p105^{Rb}$. This may be significant for head and neck cancer, as HPV6 infections are linked to papillomata, while HPV16 is associated with squamous cell carcinomas. Inhibition of the $p105^{Rb}$

by HPV16 could therefore be one of the steps leading to malignant transformation of laryngeal mucosa.

2.9.2 P53 gene

Another example of a tumor suppressor gene is the p53 encoding gene. At first it was considered as an oncogene product because of its elevated levels in most cancers. Yet p53 proved to be a product of a mutated gene, which in its unmutated state normally suppresses transformation. The gene is located on chromosome region 17p13 and codes for a 53 kD nuclear protein. Much like $p105^{Rb}$, p53 is inactivated through phosphorylation by a cdc 2 kinase in the S-phase of mitosis.

P53 is involved in downregulating S-phase activity through DNA binding. Just as with $p105^{Rb}$ an association has been described with HPV16 oncoproteins [55]. In cervical squamous cell carcinoma, HPV-positive cells showed normal Rb and p53 protein expression. In contrast, these proteins showed an altered expression in HPV-negative cells [13]. As changes in p53 gene are single base mutations, loss of heterozygosity (LOH) analysis is not sensitive enough; thus DNA sequencing is mandatory. More consistent results are obtained with immunohistochemical (IHC) detection. This reflects the fact that mutant p53 is more stable than wild-type p53. Therefore, an elevated protein level, detected with IHC, must represent the mutant form. With DNA sequencing, specific p53 mutational hotspots have been detected. These differ according to the tumor type [54,71,92].

Analogous to the Rb gene in inherited juvenile retinoblastoma, p53 mutations in the germ line of cancer-prone families with Li-Fraumeni syndrome have also been reported [75]. This supports the idea that the p53 gene is a tumor suppressor gene.
In half of the patients with head and neck cancer, p53 overexpression has been reported. Mutation of the p53 gene in HNC has also been described in various reports for approximately 50% of all patients [2,17,32,33,48,70,71,74,129].
Besides an overexpression in 67% of the patients, one study also reported a correlation with a history of heavy smoking [32,33]. In addition to a reported overexpression of p53, another study reported a concomitant high percentage of *ras* mutations and HPV infections [2].

The reports cited above show that there is little doubt about the importance of the role that tumor suppressor genes play in oncogenesis.

2.10 Miscellaneous molecular markers

Many reports have been published about prognostic factors other than the classical clinical and histological parameters. These factors represent a diverse group of immunological, serological, membrane-bound, or even intracellular cellular markers. Currently, most of these markers do not seem to be related to each other. Therefore, it is difficult to implicate the presence or increase of a certain factor in a specific step of oncogenesis. However, apart from the factors involved in the immune response, it is highly conceivable that these markers will become directly or indirectly related to oncogenes and tumor suppressor genes. The most important markers are listed in Table 2.
Nevertheless these markers may have several attractive aspects. In the future, serum markers could prove their value by creating the possibility to monitor patients in a simple and non-invasive way. Radioactive labeled antibodies to membrane-bound tumor antigens could be used for detecting small tumors or metastases; they could even be applied as a new powerful therapy for malignant tumors.

Unfortunately, at this time there are no serum markers or combinations of markers that possess sufficient sensitivity and specificity to be useful in a clinical setting. Until now, only a few antigens have been used in experimental studies trying to locate small tumor masses with the help of specific radiolabeled antibodies [93,126]. No clinical therapeutic studies involving these antigens have been reported.

Table 2. Miscellaneous molecular markers in head and neck squamous cell carcinoma.

Category	Specific markers	References
Enzymes	thymidine kinase (TK)	[38,128]
	dTTPase	[128]
	ornithine decarboxylase	[134]
	lactate dehydrogenase	[18]
Oncofetal antigens	carcinoembryonic antigen (CEA)	[18,85,128]
	others	[45,46,76]
Hormone receptors	estrogen/progestron receptor	[107,125]
	androgen receptor	[79,120,125]
Glycoproteins	CA 19-9, CA 50	[47,128]
	E-cadherin	[105]
	P-glycoprotein	[64,90,136]
	α-1-antitrypsin	[66]
	CAK1, Thomsen-Friedenreich antigen	[8,26]
	others	[12,136]
Other proteins	ferritin	[42]
	ß-2-microglobulin	[121]
Viral markers	Epstein-Barr virus	[25,27,108]
	herpes simplex virus	[108]
	papilloma virus	[44,59,61,108]
Membrane antigens	cytokeratins, vimentin	[3,86,121,124]
	ABH isoantigen	[121]
	SCC-associated antigen (SCC)	[18,20,36,128]
	E48, U36	[39,93]
	HLA antigens	[51]
	A9 antigen/α6ß4 integrin	[123,126,135]
	Ki-67	[62]
	others	[1]
Unspecified	sialic acid	[6,18]
	P15-E	[116]

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CHAPTER 3

PROTEIN TYROSINE KINASE ACTIVITY IN LARYNGEAL SQUAMOUS CELL CARCINOMA

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Summary.

Oncogenes play an important role in the process of malignant transformation. Since many of the protein tyrosine kinases (PTK) are products of oncogenes, the aim of this study was to demonstrate whether an increased PTK activity could be found in head and neck tumors. By using a non-radioactive dot-blot assay, PTK activity was measured in tumor and normal tissues of 38 patients with laryngeal cancer. The control group consisted of 19 healthy persons. PTK activity in tumor cells was significantly higher ($p < 0.001$) than in normal cells of the tumor patients and normal controls. Additionally, the PTK activity in the normal mucosa of the tumor patients was significantly higher than in the normal mucosa of the control group.

3.1 Introduction

The oncogenes are a group of transforming genes and appear to be one of the key elements in the process of proliferation of tumor cells. The progenitors of these oncogenes, the proto-oncogenes (c-*onc*), are considered to be responsible for the regulation of growth and differentiation of the normal cell. Mechanisms of transformation of proto-oncogenes into active oncogenes include chromosomal translocation, point mutation and DNA amplification [2,3]. A majority of the oncogenes are also very similar to the genes of some retroviruses capable of inducing tumors in animals (the so-called viral oncogenes, or v-*onc*). Amplification and/or enhanced expression of oncogenes have been found in both naturally occurring and experimentally induced tumors, as well as in cell lines from various tumors, including head and neck tumors [6,7,14,18].

Many of the protein tyrosine kinases (PTK) have been discovered to be oncogene products. These enzymes phosphorylate tyrosine residues of protein substrates, thereby activating those proteins ultimately leading to activation of specific genes and DNA synthesis. Phosphorylation and de-phosphorylation of enzymes is currently considered to be an important step in signal transduction and cellular proliferation and differentiation [11,24].
PTK can be broadly classified into two groups: the receptor and the non-receptor kinases.
At present, prognosis of head and neck cancer is mainly determined by the size of the primary tumor, its localization and the presence or absence of metastasis. These parameters, however, cannot predict the individual five-year survival of any patient [10]. Therefore, the search for prognostic factors and tumor markers that could more accurately discriminate between more or less aggressive tumors or predict or monitor tumor relapses, is mandatory. If accurate, these markers can be helpful in the choice of appropriate treatment [19].

Since many oncogenes code for PTK, increased or altered expressions of oncogenes may be reflected in increased PTK activity. In mammary carcinoma and more recently in gliomas and astrocytomas a correlation has been found between the level of PTK activity and the disease-free survival of patients with these tumors [5,8].

The aim of the present study was to determine if a difference could be established between PTK activity in squamous cell carcinoma of the larynx and that in normal mucosa.

In this paper we present the results of PTK activity in the cytosolic and membrane fractions of homogenates of laryngeal cancers, of non-tumorous laryngeal mucosa of patients with laryngeal cancer and of mucosa of control patients.

3.2 Materials and methods

3.2.1 Clinical material

Fresh biopsies were taken from the surgical specimen by the pathologist immediately after laryngectomy. From each surgical specimen two biopsies were taken: one from a representative part of the tumor and the other from a non-tumorous area, mostly near the resection margin. To confirm this so-called non-tumorous state, this latter biopsy was taken adjacent to those taken for histological confirmation of the adequacy of surgical resection margins. Additional specimens were also obtained during staging diagnostic endoscopy procedures by dividing the routine diagnostic biopsies taken. In a few patients specimens were also sampled from a non-tumorous part of the larynx.

Histological diagnosis was according to WHO criteria. Patients were staged according to the TNM classification [9]. In all, specimens were obtained from 38 patients with squamous cell carcinoma of the larynx. Non-tumorous laryngeal mucosa was also collected from 29 of the patients. Additionally, 19 biopsy specimens of normal mucosa were obtained as "controls" from patients undergoing uvulopalatopharyngoplasty. Ten of these control patients were smokers. All tissues were stored at -80° C until use in the protein tyrosine kinase assay.

3.2.2 Cell fractionation

At least 10 mg of tissue was necessary for the whole PTK assay (usually between 30 and 60 mg was obtained from each patient). Tissues were thawed and then lyzed with a mechanical homogenizer (Omni 1000, Omni International, Waterbury, CT, USA) using two volumes of an extraction buffer containing 10 mM TRIS-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 0.5 mg/ml aprotinin. Suspensions were centrifuged for 10 min at 800 *g* in order to remove any cellular debris and nuclei. Supernatants were then centrifuged for 60 min at 48,000 *g*. The resulting supernatants (referred to the "cytosolic fraction") were used to determine cytosolic PTK activity. The remaining pellets were resuspended in 200 µl of a buffer solution containing 50 mM TRIS-HCl (pH 7.5), 20 mM Mg-acetate, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, 1 mM dithiothreitol, 30 mM Na_3VO_4 to which 0.5%

Nonidet P-40 was added. After sonication (2 x 10 s) suspensions were centrifuged at 48,000 *g* for 60 min. The resulting supernatants (the solubilized membrane fraction) were used to determine the membrane-bound PTK activity.

3.2.3 Determination of protein concentrations

The protein content of the cytosol and solubilized membrane fractions were determined according to the method of Lowry et al. [12] A bovine serum albumin solution diluted in extraction buffer and in solubilization buffer, respectively, was used as a standard. If contamination of the fractions with blood was suspected, the protein content was corrected following spectrophotometric determination of the hemoglobin concentration in the fractions examined. In previous studies the PTK activity of red blood cells was found to be insignificant [5].
The mean protein content was 6.6 ± 4.2 mg/ml (range 0.8-12.6 mg/ml).

3.2.4 Determination of PTK activity

The PTK activity of the fractions was determined by a non-radioactive dot-blot assay as described by Rijksen et al. [17]. This method is based on the detection of phosphorylated tyrosine residues in an artificial substrate consisting of a 4:1 ratio of poly - glutamic acid to tyrosine (PGT) by using monoclonal antibodies to phosphotyrosine (IG2; Oncogene Science, Manhasset, N.Y., USA). All samples were phosphorylated in duplicate and then tested in duplicate, resulting in four values for each sample. Variance was less than 10% for each sample. The PTK activity was then expressed as picomoles tyrosyl residues which are phosphorylated in PGT per minute per milligram of protein (=pmol/min.mg protein).

3.2.5 Statistical methods

To evaluate differences in PTK activity two-sample *t*-tests and paired *t*-tests were applied as appropriate. To ensure variance stabilization the logarithm of the measurements was taken. The SPSS statistical software program was used throughout.

3.3 Results

PTK activities in the cytosolic and membrane fractions of the squamous cell carcinomas of the larynx and non-tumorous laryngeal mucosa from the cancer patients and normal controls persons are shown in Table 1.

3.3.1 Cytosolic PTK activity

The mean values of cytosolic PTK activity of the laryngeal cancer samples were 5.7 times higher than that of the normal mucosa of the control group ($p < 0.001$). The mean cytosolic PTK activity of the tumor samples was also 1.9 times higher than that of the non-tumorous laryngeal mucosa of the same patients ($p < 0.001$). Additionally, non-tumorous laryngeal mucosa of patients with laryngeal cancer showed a three-fold increase in mean cytosolic PTK activity compared to normal mucosa of the control group ($p < 0.001$). No differences in mean cytosolic PTK activity could be observed when the control group was divided into smokers and non-smokers (49 ± 28 vs 46 ± 23, 95% confidence interval 76-19).

Age and/or sex did not influence PTK activity. No differences in PTK activity could be found when the laryngeal cancer group was separated into glottic and supraglottic tumors. Cytosolic PTK activity of samples taken from recurrent tumor failing prior irradiation tended to be lower than those of primary tumors (Table 2). These differences were not significant ($p=0.290$).

Only 3 of the 46 patients had well-differentiated carcinomas, while the remaining tumors showed moderate or moderate to poor differentiation. However, no differences were found in cytosolic PTK activity based on histological differentiation. Grouping the patients according to their TNM classification or stage also did not result in any statistically significant differences.

Table 1. Mean protein tyrosine kinase activity (in pmol P-tyr/mg protein.min) in tumor samples, non-tumorous laryngeal mucosa samples and normal control tissue

Tissue		Cytosolic (mean ± SD)	Membrane-bound (mean ± SD)
Tumor	(n=38)	246 ± 167	1315 ± 1180
Non-tumor	(n=29)	128 ± 83	842 ± 1126
Control	(n=19)	48 ± 25	568 ± 322

3.3.2 Membrane-bound PTK activity

The PTK activity of the membrane fractions of the tumors was also increased when compared to non-tumorous laryngeal mucosa and normal control mucosa (Table 1). However, due to the large variations in membrane-bound PTK activity found in the different samples, we did not find any significant differences among the three

groups. Further, no correlation was found between these activities and stage, histology and/or previous irradiation.

3.4 Discussion

As noted previously, PTK activity has been associated with products of some oncogenes and growth factor receptors, while the major part of PTK activity is supposed to be associated with oncogene expression. This suggests that an increase in PTK activity may reflect an increase of oncogene activation or cell proliferation and might be a useful parameter for evaluating malignant lesions. In mammary carcinoma variable expression of several oncogenes with intrinsic PTK activity has been reported [22]. Remarkably, however, PTK activity in cancers of the human breast has been increased in all cases when compared to benign mammary tumors and normal breast tissue [8,15]. Additionally, a relation between the level of cytosolic PTK activity, tumor grade and prognosis has been found in mammary carcinoma, astrocytoma and glioma [5,8].

Several oncogenes and growth factor receptors have been found in squamous cell carcinoma of the head and neck, but to our knowledge no one else has investigated the expression of oncogenes with PTK activity in squamous cell carcinoma of the larynx. In other sites, Field et al. [6] found an increased expression of H-*ras*, K-*ras* and c-*myc*. Merritt et al. [14] demonstrated an increase in *int*-2 and c-*myc* expression. An increased expression of c-*myc* was also found by Rivière et al. [18]. However, these oncogenes found in head and neck squamous cell carcinomas are not directly associated with PTK activity.

In contrast Rydell et al. [20] showed that the cytosolic PTK activity of tumors of the oral cavity was sixfold higher when compared to normal oral mucosa. Although obtained by a different method, these data fully support our data.

Many authors have reported an increase in epidermal growth factor receptors (EGFr) in squamous cell carcinoma of the head and neck [1,4,18,21]. EGFr also has an intracellular PTK activity. Stimulation of the EGFr with EGF in a squamous cell carcinoma cell line of the head and neck has been found to increase autophosphorylation of the receptor [13,16]. Increased cytosolic PTK activity after EGF stimulation has been reported as well [13].

An intriguing finding in our present study is that PTK activity in histologically normal laryngeal mucosa of tumor patients is increased when compared to control

tissues from healthy persons. These results suggest an enzymological change in the non-tumorous laryngeal mucosa of patients and may be significant, since these patients have a higher risk for developing a second tumor. In Terhaard's [23] 1991 University thesis, after 10 years patients with laryngeal carcinoma have a 30% cumulative probability for developing a second squamous cell carcinoma of the head and neck or of the lung.

As all of our tumor patients were moderate to heavy smokers we could not compare the PTK activity in tumor and non-tumorous tissues of smoking and non-smoking patients. However, the increased PTK activity in histologically normal laryngeal mucosa of our tumor patients could not be attributed to smoking, as we found no differences in PTK activity between smoking and non-smoking control patients.

All of our control biopsies were taken from mucosa of the palate. The differences in PTK activity could not be explained by the different location of the samples, as the PTK activity of healthy trachea and larynx mucosa was the same as that of the palate (data not shown).

Bergler et al. [1] reported that non-tumorous mucosal specimens from patients with squamous cell carcinomas of the head and neck had positive staining for EGFr (which has a PTK activity), as did tumor samples from the same patients. Patients with heavy smoking and drinking habits without tumor also showed an EGFr expression, while non-smoking patients hardly showed any staining. Their results also demonstrated changes in the biochemical properties of the cell before any morphological changes could be observed. However, this could be related to patients' smoking and drinking habits.

It is also possible that the increase in PTK activity was the result of paracrine stimulation of normal mucosa by tumor. However, our non-tumorous laryngeal samples were taken at resection margins which were at least several centimeters from proven tumor.

We could not find a significant difference in PTK activity when we grouped patients according to tumor size, nodal status, histological grade, sex or age. However, after calculation of the ratio between the cytosolic PTK activity of the tumor and that of the non-tumorous laryngeal specimen (the ' T/N ratio ') we became aware of a certain trend. Patients which had tumor recurrences after irradiation showed a smaller T/N ratio than did those treated for a primary tumor alone (Table 2).

Table 2. Tumor/Non-tumor (T/N) ratio of cytosolic PTK activity in laryngeal mucosa: mean values for patients treated only with primary surgery (primary) and for previously irradiated patients (recurrence)

	Tumor (mean ± SD)	Non-tumor (mean ± SD)	T/N Ratio (mean ± SD)
Primary (n=18)	279 ± 156	116 ± 74	3.30 ± 2.45
Recurrence (n=11)	189 ± 142	146 ± 94	1.91 ± 2.00

This difference was partly due to an increase in the PTK activity of the non-tumorous mucosa in the previously irradiated patients. However, this increase in PTK activity in the non-tumorous laryngeal mucosa cannot be explained by an increase in scar tissue due to radiotherapy. Such scar tissue is filled with fibrin and fibroblasts, which increases the absolute protein content of the sample. As enzyme activity is expressed per milligram of protein, only a decrease (in the specific activity) should have been expected. On the contrary, an increase was observed in PTK activity. At this moment we are unable to give an explanation for these differences in the T/N ratio, but it is possible that it reflects a higher potential of tumor recurrence or secondary tumor development.

As the follow-up period in our patient group is still relatively short we were not able to look for a correlation between PTK activity and a disease-free interval. However, if this relation is confirmed for our patients, it could have important implications for treatment modalities considered. Our present results show that the determination of PTK activity may be significant for understanding tumor oncogenesis. Whether this implies true clinical value needs further investigation.

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CHAPTER 4

PROTEIN TYROSINE (DE-)PHOSPHORYLATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Summary

Protein phosphorylation plays an important role in signal transduction of both normal and neoplastic cells. Since increased protein tyrosine phosphorylation may be associated with malignant transformation, we studied the activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPase) in patients with various head and neck tumors. Furthermore, we determined the patterns of tyrosine phosphorylated protein (P-tyr) in tissues by Western blotting. Enzyme activities were studied in tumor and, histologically proven, non-tumorous tissues of 54 patients and in 11 controls. P-tyr patterns were determined in three patients and two controls. PTK and PTPase activities were significantly increased in tumor tissues compared to normal tissue of the cancer patients as well as controls. P-tyr levels in tumors were higher than in normal tissues. Additionally, PTK activities in normal tissue of tumor patients were significantly higher than in normal tissue of the control group. The same trend was observed for the PTPase activity and P-tyr levels.

4.1 Introduction

The state of phosphorylation of tyrosyl residues in proteins plays a crucial role in regulating cell growth and differentiation. The steady state level of tyrosine phosphorylation of cellular proteins depends on the balance of the action of protein tyrosine kinases (PTK; ATP: protein-tyrosine O-phosphotransferase, EC 2.7.1.112) and protein tyrosine phosphatases (PTPase; protein-tyrosine-phosphate-phosphohydrolase, EC 3.1.3.48). Both receptor (e.g. epidermal growth factor receptor (EGFr)) and non-receptor (e.g. *src* family) PTKs have been identified, while a number of tyrosine kinases appear to function as oncogenes. As is the case for PTKs, PTPases constitute a family of intracellular and transmembrane proteins [4].
Alteration in the regulation of the phosphorylation state of tyrosyl residues on various cellular proteins appears to be an important pathway towards neoplastic transformation [1,19]. This alteration may arise through either deregulation or overexpression of a PTK or the underexpression of a PTPase.

Amplification or expression of mutated forms of oncogenes with PTK activity has been directly associated with neoplastic growth [19] and is sometimes related to the clinical behavior of certain tumors and thus to the prognosis of the patient [3,16]. The relationship between PTK activity and the growth and/or differentiation of tumor cell lines and/or human tumors has been described by several authors [2,5,15,20]. Less research in this respect has been done on the PTPases. Further characterization of the role and regulation of PTK and PTPase activity in tyrosyl phosphorylation could provide clues for the understanding of the complex process of signal transduction during cell growth and differentiation of both normal and neoplastic cells.

Recently we have reported on PTK activity in laryngeal cancer [20]. We observed a significant increase in cytosolic PTK activity in tumor tissue when compared to normal tissue of cancer patients as well as controls. Moreover, we found a significant increase in PTK activity in normal mucosa of cancer patients compared to controls, suggesting an enzymologically "premalignant" state of the non-tumorous mucosa of the cancer patients.

In this paper we extend these data on cytosolic PTK activity by including a larger group of patients with different kinds of tumor localizations in the upper aero-digestive tract. We have also measured PTPase activity in tumorous and in non-

tumorous mucosa of these patients, and compared those to PTPase activity in corresponding mucosa of healthy individuals. As a read-out of combined PTK and PTPase activity, we analyzed the tyrosyl phosphorylation state of substrate proteins in tumorous, non-tumorous and control mucosa by using western blotting with an anti-phosphotyrosine-specific antibody.

4.2 Materials and methods

4.2.1 Clinical material

Tumor tissue was obtained from 54 patients with squamous cell carcinoma of the upper aero-digestive tract. Primary sites are shown in Table 1. Tissue samples were taken from the surgical specimen by the pathologist immediately after resection. From each surgical specimen two samples were taken: one from a representative part of the tumor and the other from a histologically proven, non-tumorous area, mostly near resection margins. Samples were also obtained during staging diagnostic endoscopic procedures by dividing diagnostic biopsies.

Table 1. Primary sites and stages of tumors studied for protein (de-)phosphorylation activity.

Primary site	Stage	Cases
Larynx (*n*=45)	1	11
	2	16
	3	9
	4	9
Hypopharynx (*n*=2)	4	2
Nasopharynx (*n*=1)	4	1
Oropharynx (*n*=3)	2	2
	4	1
Tongue (*n*=3)	2	1
	3	2

In a few patients samples were also taken from a non-tumorous part of unrelated mucosa. In addition 11 control samples were obtained from patients without cancer who underwent elective surgery for other causes (i.e. 9 uvulopalatopharyngoplasties,

one tracheotomy and one Zenker's diverticulectomy). All tissues were stored at -80°C until use in the different assays.

Histological diagnosis was on World Health Organization criteria. Only four of the 54 patients had well differentiated carcinomas and two had poorly differentiated tumors. The others showed moderate (n=40) or moderate to poor (n=8) differentiation. Non-tumorous mucosa was also collected from 38 patients. Patients ages varied from 32 to 88 years (mean, 62.7 years for the cancer patients and 49.6 years for the control patients). Six of the cancer patients and three of the control patients were female. Tumor specifications (TNM classification, localization and stage) were made according to UICC classifications [6]. As shown in Table 1, there were 11 stage 1, 19 stage 2, 11 stage 3 and 13 stage 4 patients. Thirteen patients had lymph node metastases. Additionally, three tumor, three non-tumor and two control samples were obtained for the study of tyrosyl-phosphorylated proteins by immunoblot assay.

4.2.2 Cell fractionation

At least 10 mg of tissue was necessary for assays (but between 30 and 60 mg was usually obtained). The samples were thawed and then lyzed with a mechanical homogenizer (Omni 1000, omni International, Waterbury, Conn., USA) in two volumes of extraction buffer. Samples used for PTK assay and for immunoblotting experiments were extracted in buffer containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 50 KIU/ml aprotinin. Samples for the PTPase assay were extracted in 50 mM HEPES (pH 7.5), 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol. The suspensions obtained were centrifuged for 10 min. at 800 g in order to remove cell debris and nuclei. Supernatants were then centrifuged for 60 min. at 48,000 g. The resulting supernatants (referred to as "cytosolic fraction") were used to determine cytosolic PTK and PTPase activity or used in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting.

4.2.3 Determination of protein concentration

The protein content of the cytosolic fractions was determined according to the method of Lowry [9]. A bovine serum albumin solution diluted in water was used as standard. If contamination of the fractions with blood was suspected, protein content was corrected by spectrophotometric determination of the hemoglobin concentration in the fractions. We thereby assumed that PTK activity of red blood cells was not significant [2].

The mean protein content of the cytosolic fraction was 6.6 mg/ml (range, 0.8-12.6 mg/ml, S.D.= 4.2).

4.2.4 Determination of protein tyrosine kinase activity

The PTK activity of the fractions was determined by a non-radioactive dot-blot assay as described by Rijksen et al. [11]. This method is based on the detection of phosphorylated tyrosine residues in an artificial substrate using 4:1 poly glutamic acid:tyrosine (PGT, Sigma, St. Louis, Mo., USA) and a monoclonal antibody to phosphotyrosine (IG2, Oncogene Science, Manhasset, N.Y., USA). All samples were phosphorylated in duplicate and detected in duplicate, resulting in four values for each sample. Variance was less than 10% for each sample. The PTK activity was expressed as picomoles tyrosyl residues which are phosphorylated in PGT per min. per mg of protein (=pmol P-tyr/min.mg protein).

4.2.5 Determination of protein tyrosine phosphatase activity

Prior to the determination of PTPase activity, a radio-labeled PTPase substrate was prepared. PGT (5 mg) was phosphorylated in vitro by PTKs extracted from A431 cells (1 mg) in a buffer containing 50 mM HEPES (pH=7.2), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM NaF, 1 mM EDTA/EGTA, 1 mM dithiothreitol, 100 µM orthovanadate and 100 µM ^{33}P-ATP with a specific activity of 0.15 Ci/mmol for 20 h at 20°C. PGT was precipitated in trichloroacetic acid (TCA). The precipitate was collected by centrifugation and washed with ice-cold TCA to remove unincorporated ^{33}P-ATP. The final precipitate was resuspended in 1 M NaOH and the remaining unincorporated ^{33}P-ATP was separated from labeled PGT on a Sephadex G-25 PD 10 column (Pharmacia, Uppsala, Sweden). The column was eluted with a buffer containing 50 mM HEPES (pH 7.5), 0.1 mM EDTA and 1 mM dithiothreitol. ^{33}P-labeled PGT was used as substrate for PTPases at a concentration of 0.25 mg/ml and a ^{33}P-phosphate incorporation of 5 mmol per mole of tyrosine.
Dephosphorylation of ^{33}P-labeled PGT was carried out at 37°C in a total volume of 40 µl containing 50 mM HEPES (pH 7.5), 0.1 mM EDTA and 1 mM dithiothreitol. The reaction was started by adding cytosolic fractions of the samples (2 µg/assay). After 10 min the reaction was stopped by the addition of 200 µl 12 % TCA. Radio-labeled PGT was precipitated and after centrifugation at 14,000 *g* for 10 min, the amount of liberated ^{33}P-PO_4 in the supernatant was quantified by liquid scintillation counting. PTPase activity was expressed as pmol P-tyr/min.mg protein.

4.2.6 Determination of tyrosyl phosphorylated proteins

Eight samples were analyzed: three tumors, three non-tumorous mucosa and two controls. Phosphorylation was carried out by adding 500 µM ATP (in 10 µl incubation buffer, see above) to 100 µg of cytosolic fractions (in 40 µl extraction buffer). Samples were phosphorylated for 60 min at 37°C both in the presence and

absence of 1 mM orthovanadate (in 10 µl incubation buffer). The reaction was stopped by adding 20 µl sample buffer (final concentrations: 60 mM Tris-HCl (pH 6.8), 50% glycerol, 2% SDS and 0.005% bromophenol blue). After boiling samples for 5 min, SDS-polyacrylamide gel electrophoresis was carried out on a 3% stacking gel and a 10% separating gel. After electrophoresis [8], proteins were transferred electrophoretically to a polyvinylidene difluoride filter and blocked with 5% bovine serum albumin in phosphate-buffered saline. Membranes were probed with monoclonal mouse antibody directed against P-tyr (PY20, ICN Biomedicals, Costa Mesa, CA, USA) (2 µg/ml incubation buffer with 1% normal goat serum). Immune complexes were visualized with an immunogold-silver-staining method [11].

4.2.7 Statistical methods

To evaluate differences in PTK and PTPase activity analysis of variance was applied as appropriate. To ensure variance stabilization the logarithm of the measurements was taken. The SPSS statistical software program was used throughout (SPSS Inc, Chicago, IL, USA).

4.3 Results

4.3.1 Enzyme activities

Mean PTK and PTPase activities of the cytosolic fractions of tumorous, non-tumorous and control samples are shown in Table 2. The mean value of PTK activity of the tumor samples was 5.3 times higher than those of the normal mucosa of the control group ($p < 0.001$) and 2.4 times higher than that of the non-tumorous mucosa of the same patients ($p < 0.001$). Non-tumorous mucosa from the patients with head and neck cancer showed a 2.2 fold increase in mean PTK activity over normal mucosa of the control group ($p = 0.003$).

The mean value of PTPase activity of tumor samples was 1.8 times higher than that of normal mucosa of the control group ($p = 0.016$) and 1.4 times higher than that of the non-tumorous mucosa of the same patients. Non-tumorous mucosa from the patients with head and neck cancer showed a slight increase in mean PTPase activity when compared to normal mucosa of the control group, but this difference was not significant. Age and/or sex did not influence PTK or PTPase activity. Grouping patients according to their TNM classifications, localizations, stages and/or differentiations, also did not result in any statistically significant differences in enzyme activity.

Table 2. PTK and PTPase activities and PTK/PTPase ratios in tumorous and non-tumorous mucosa of cancer patients and normal control mucosa. The values are mean ± SD. PTK and PTPase activities are expressed in pmol P-tyr/min.mg protein.

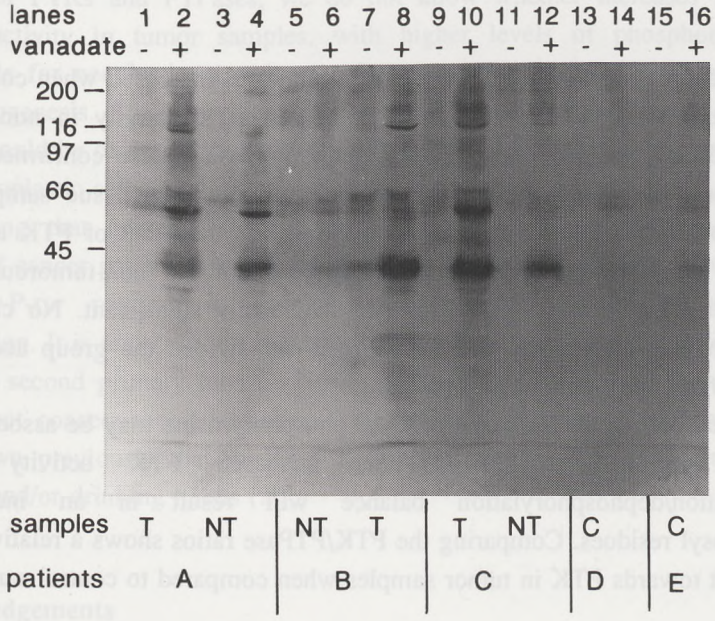
	Tumor (n=54)	Non-tumor (n=38)	Control (n=11)
PTK activity	258 ± 164	107 ± 70	48 ± 28
PTPase activity	424 ± 213	311 ± 213	238 ± 106
PTK/PTPase ratio	0.70 ± 0.46	0.45 ± 0.25	0.24 ± 0.19

The ratio between PTK and PTPase activity was also used as a means to evaluate tyrosine phosphorylation state of proteins (PTK/PTPase ratio, Table 2). The mean PTK/PTPase ratio of tumor samples was 2.9 times higher than that of the ratio of control samples ($p < 0.0001$) and 1.6 times higher than that of the non-tumorous mucosa of the same patients ($p < 0.001$). Non-tumorous mucosa of patients with head and neck cancer showed a 1.7 fold increase in mean PTK/PTPase ratio when compared to normal mucosa of the control group ($p = 0.004$). However, no direct correlation was found between the ratios in tumor and non-tumor tissue ($r= 0.303$).

4.3.2 Levels and patterns of tyrosyl phosphorylated proteins

As a direct measurement of the balance between PTK and PTPase activity we investigated the pattern of tyrosine phosphorylated proteins in the cytosolic fractions of tumorous, non-tumorous and control samples. Phosphorylation of endogenous proteins, with subsequent electrophoresis and immunoblotting revealed several phosphotyrosyl containing proteins with apparent molecular weights ranging from 33 to 120 kD (Fig.1). The effects of orthovanadate, a PTPase inhibitor, were clearly seen in the lanes where phosphorylation was done in the presence of vanadate (+) when compared to those lanes where vanadate was omitted (-). All bands essentially disappeared when orthovanadate was omitted during phosphorylation. The intensity of corresponding bands was higher in tumor than in non-tumor in all patients (lanes 2/4, 8/6 and 10/12). In the three non-tumor samples the 105 kD band disappeared. In one non-tumor sample (patient C) the p53 kD band was also absent. The intensities of the corresponding bands of the non-tumor samples were higher than those of the control samples (with hardly any bands visible in the latter).

Figure 1. Immunoblot of cytosolic fractions of samples of patients A, B and C and controls D and E. The lanes show bands representing endogenous proteins with tyrosyl phosphorylation, in the presence (+) or absence (-) of vanadate, in tumor (T) samples, non-tumor (NT) samples and control (C) samples. The position of molecular markers (in kilodaltons) is shown on the left.



4.4 Discussion

PTK activity in head and neck cancer has been investigated previously [15,20]. Results showed that cytosolic PTK activity of tumors was increased when compared to that in control and non-tumorous mucosa of the upper aero-digestive tract. Remarkably, PTK activity of non-tumorous tissue of cancer patients was also increased when compared to control mucosa of healthy patients [20]. This altered enzyme activity may reflect an ultrastructural premalignant state of non-tumorous mucosa of patients at high risk for developing a second tumor. A paracrine effect is less likely due to the large distance between tumor and non-tumor samples.

As the extent of tyrosyl phosphorylation is the result of both phosphorylation ánd dephosphorylation, we were interested in the (relative) activity of both PTKs and PTPases. To our knowledge this is the first report describing PTPase activity in

squamous cell carcinomas of the head and neck. PTK activity is associated with oncogene products and PTPases are believed to counteract the effects of increased PTK activity. Many authors have described an increase in PTK activity or an increased expression of PTK-related oncogenes in a wide variety of cancers, such as mammary carcinoma [5,10,17], brain tumors [2] and head and neck tumors [13-15,20].

Our results clearly show that PTK activity in tumors is increased when compared to that in control mucosa. The finding of increased PTK activity in non-tumorous mucosa in cancer patients compared to control mucosa is also confirmed. PTPase activity is also increased in both tumor and non-tumorous tissue samples when compared to normal mucosa but this increase is less than that of PTK activity. A trend could be observed of increased PTPase activity in non-tumorous samples compared to controls although it was not statistically significant. No clear differences in enzyme activities could be found if we divided the group according to different tumor and/or patient parameters.
As mentioned before, an increased level of phosphotyrosine may be associated with neoplastic growth. A shift towards increased PTK activity in the phosphorylation/dephosphorylation balance will result in an increase in phosphotyrosyl residues. Comparing the PTK/PTPase ratios shows a relative enzyme activity shift towards PTK in tumor samples when compared to control samples.

We also tried to evaluate the balance between PTK and PTPase activities by determining the amount of phosphotyrosyl residues on western blot analysis. However, meaningful results were only obtained when PTPase activity was completely blocked by the addition of the PTPase inhibitor orthovanadate. In the absence of vanadate no phosphotyrosyl-containing proteins could be detected, neither in tumor nor in non-tumor or control tissues. Obviously, in vitro PTK activity is overruled by PTPase activity. If, in in vivo experiments, the dephosphorylation of phosphotyrosine is prevented by the use of orthovanadate, cellular phosphotyrosine increases and certain cells will exhibit a transformed phenotype or different growth characteristics [7,12]. Taken together, these data indicate that in vivo PTPase activity is likely to be under rigid control. PTPases appear to counteract the oncogenic effects of PTKs and therefore may function as anti-oncogenes. They are therefore enzymes whose encoding genes could possibly act as tumor-suppressor genes [4,18].
In contrast, PTPase activity is obligatory for the activation of *c-src* kinases. Consequently, the putative PTPase involved in the dephosphorylation of *c-src* might

be considered an oncogene. Indeed, phosphorylation/dephosphorylation is an important regulatory mechanism which appears to be very complex. A change in enzyme activities could reflect an increase or decrease in the activation of genes responsible for the control of cell growth and differentiation by means of a change in the level of phosphorylated tyrosyl residues. As we did not identify the target proteins of PTKs and PTPases, we do not know whether increases in PTK and PTPase activity in tumor samples, with higher levels of phosphotyrosine, are responsible for neoplastic growth or only represent concurrent changes associated with oncogenesis. Still, the identification of PTKs and PTPases and their target proteins could be of major importance in understanding their role in signal transduction in neoplastic cells.

The finding that enzyme activities and phosphotyrosyl levels in non-tumorous mucosa of cancer patients are elevated compared to healthy control mucosa and the different P-tyr patterns in tumor and non-tumor tissues warrants further investigation. It is tempting to speculate that this increase in enzyme activity may be related to second primary tumors. However, we do not believe that these changes are a direct consequence of tobacco and/or alcohol consumption alone, since we have shown previously that the PTK activity in control mucosa is unaffected by smoking and/or drinking habits [20].

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CHAPTER 5

HISTOCHEMICAL DETECTION OF PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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(submitted for publication)

Summary

Protein tyrosine phosphatases (PTPases) catalyze the dephosphorylation of phosphotyrosyl residues of proteins. PTPases are supposed to counteract the protein tyrosine kinases (PTKs) in the control of the equilibrium between phosphorylation and dephosphorylation. Specific PTPases can also activate certain PTKs, and may therefore be considered as oncogenic. Increased activity of specific PTPases may also be important in the activation of signaling pathways in malignant processes. Whereas a large body of knowledge exists with regard to the role of PTKs in cell transformation and oncogenesis, little is known about the exact role of PTPases in these particular processes.

In this chapter we investigated whether the PTPase activity could be detected with an enzymehistochemical technique. We found that PTPase activity was easily detected with light microscopy. We found increased PTPase activities in tumor cells. To a lesser extent, PTPase activities were elevated in non-tumor tissue compared to control tissue. We could not detect any differences in staining intensity related to grade, stage, previous radiotherapy, or site. We also found high activities in the surrounding connective tissue. This activity was mainly localized in the collagens, not in the fibroblasts and must be considered as an artefact. Orthovanadate, a PTPase inhibitor, strongly inhibited the enzyme activity. No staining was seen in the absence of phosphotyrosine or in the presence of phosphoserine or phosphothreonine. Therefore this method must be considered as specific for PTPase activity.

5.1 Introduction

Phosphorylation and dephosphorylation of proteins play a major role in the signal transduction leading to cell proliferation and differentiation of normal and malignant cells [4,18]. Phosphotyrosine-protein phosphatases (or: protein tyrosine phosphatases, PTPases) catalyze the dephosphorylation of phosphotyrosyl residues, whereas the phosphorylation of tyrosine in proteins is catalyzed by protein tyrosine kinases (PTKs). Many oncogene products possess PTK activity; hence, this enzyme activity can be considered as a parameter of oncogene activation [17,19]. PTPases are supposed to counteract the PTKs in the control of the equilibrium between phosphorylation and dephosphorylation. However, recent reports also suggest that specific PTPases can activate certain PTKs, and may therefore be considered as oncogenic [24]. Increased activity of specific PTPases may also be important in the activation of signaling pathways in malignant processes. Whereas a large body of knowledge exists with regard to the role of PTKs in cell transformation and oncogenesis, little is known about the exact role of PTPases in these particular processes.

Increased expression of oncogenes with PTK activity has been found in many tumors. An increase in PTK and/or PTPase activity has been found in breast cancer, neural tumors, and head and neck cancer [3,5,11,16,20-22]. Enzyme histochemical studies in breast cancer have demonstrated an increased PTPase activity in the cytoplasm and on the membranes of the tumor cells, as well as in the surrounding connective tissue [7,10,13]. According to the authors, PTPase could play a role in the infiltrative growth or metastasis of these tumors. Increased PTPase activity in the surrounding connective tissue may reflect the production of tumor factors by the tumor cells; these factors could be responsible for the malignant behavior of cancer cells.

A previous study demonstrated that tumor tissue displays higher PTK and PTPase activities in biochemical assays than either non-tumorous tissue of the same patients or tissue of control patients. Furthermore, compared to control tissue, both enzymes in non-tumorous mucosa of cancer patients showed higher activities, indicating an enzymologically premalignant condition of the non-tumorous tissue [20,22].
In this report, the presence of PTPase activity in head and neck cancer was determined histochemically. PTPase activity was localized and (semi-)quantified in both tumor specimens and controls.

5.2 Materials and Methods

5.2.1 Patient material

Tumor tissue was obtained from 10 patients with squamous cell carcinoma of the upper aerodigestive tract. Tissue samples were taken from a representative part of the surgical specimen, immediately after resection. In addition, control samples were obtained from two patients during elective surgery for uvulopalatopharyngoplasty. All tissues were stored at -80°C until use. Histological diagnosis was according to World Health Organization criteria. All samples were moderately or poorly differentiated squamous cell carcinoma. Tumor specifications (TNM classification, localization, and stage) were made according to UICC [6]. Eight tumors were laryngeal; one was taken from the tongue and one from the tonsillar region. Six patients had been treated previously by radiotherapy (see Table 1).

5.2.2 Enzyme histochemistry

Lightmicroscopical detection of PTPase was carried out according to the method of Partanen and Pekonen, as modified by Kidd et al. [7,13]. Briefly, 6-8 µm cryostat sections were air-dried, and incubated for 4 hours at 37°C in incubation medium. This medium consisted of 10 mM O-phospho-L-tyrosine (P-tyr, Boehringer, Mannheim) and 1.75 mM $Pb(NO_3)_2$ in 50 mM MES (2-{N-morpholino} ethane sulphonic acid) buffer, pH 6.0. The sections were then washed in deionized water, four times for 5 min each and developed for 1 min in 0.2% ammonium sulphide. This was followed by washing in deionized water, four times for 5 min each. The slides were counterstained with Mayer's hematoxylin for 5 min, washed in running tap water for 10 min, dehydrated in ethanol, cleared in xylene, and mounted with DePeX (Klinipath, Zevenaar, The Netherlands). Controls included: 1) substitution of the substrate by 10 mM phosphoserine (P-ser) or 10 mM phosphothreonine (P-thr); 2) incubation without substrate; 3) enzyme inhibition by 75 µM sodium orthovanadate, a specific PTPase inhibitor; 4) incubation for alkaline phosphatase [2] and acid phosphatase activities [9].

5.3 Results

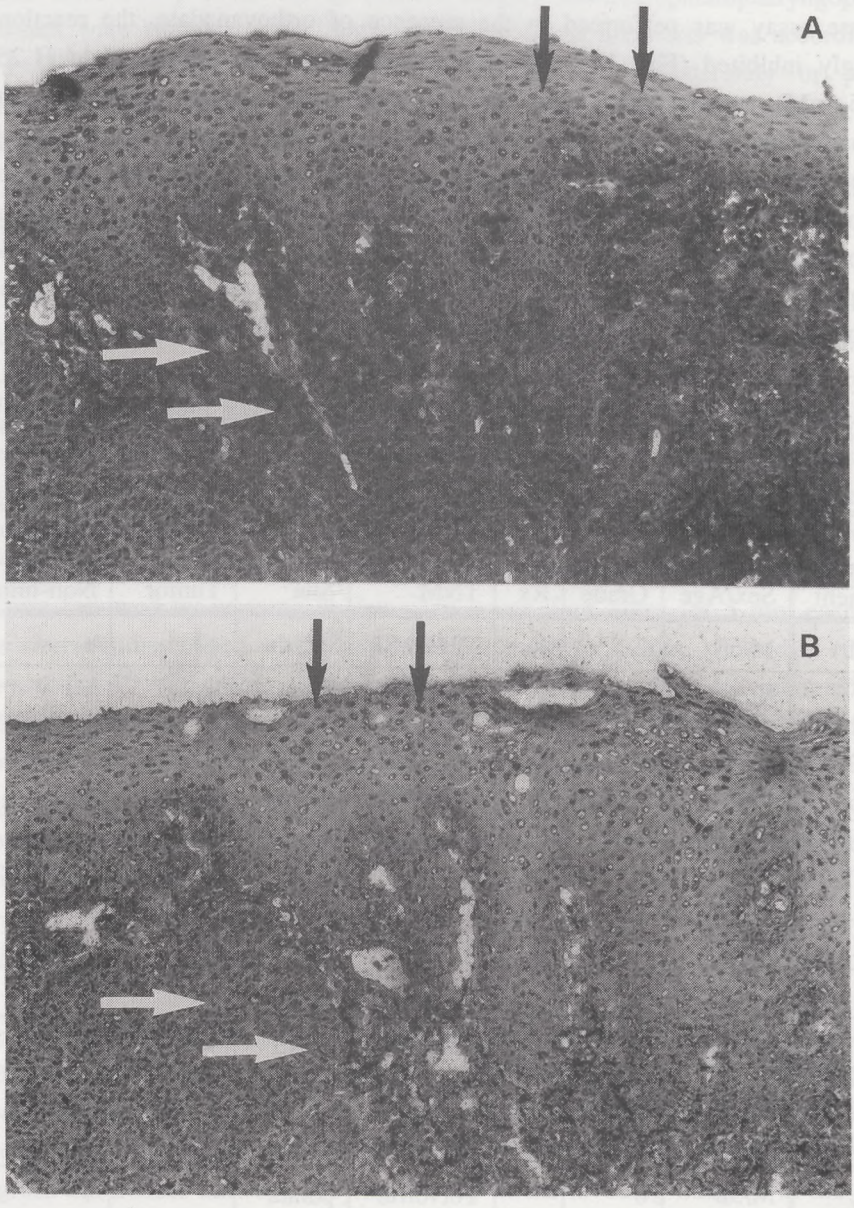
We observed moderate to strong PTPase activity in carcinoma cells as well as in the hyperplastic but benign epithelium contiguous to the tumor (Fig 1A). Staining intensities were heterogeneous in tumor tissues. Consequently, the differences between tumor and hyperplastic mucosa were sometimes small. We did not observe any gross differences between enzyme activity in the invading margins of the tumor

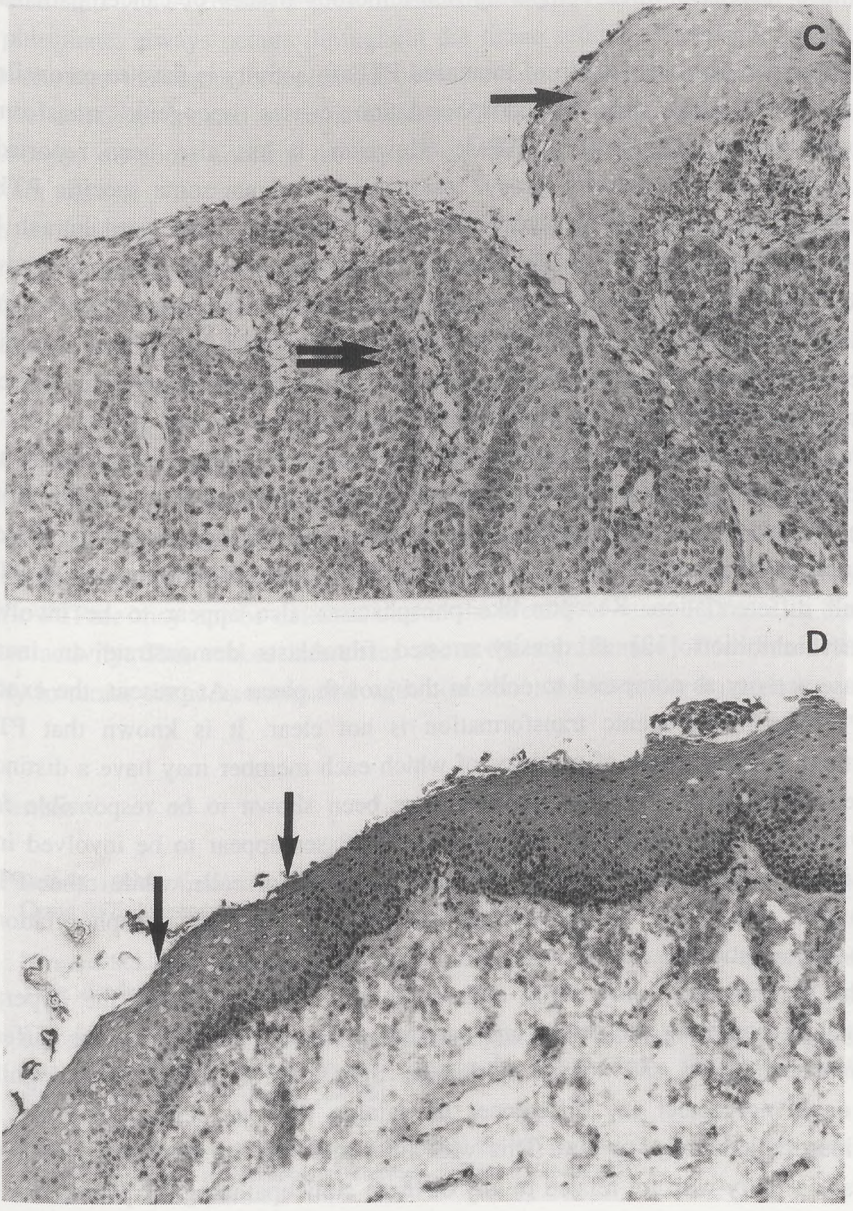
and activity in its central parts. No differences in activity were noticed between discrete tumor sites, nor in regard to histological grade, TNM stage, or previous radiotherapy (Table 1). Strong staining was seen in the connective tissue bordering the carcinoma cells, while moderate staining could be observed in more distant parts of the connective tissue. In normal, non-keratinizing squamous cell epithelium, a weak staining was observed in the cytoplasm, in the nucleus, and on the membranes (Fig. 1D). The surrounding connective tissue was moderately stained. When the PTPase assay was performed in the presence of orthovanadate, the reaction was strongly inhibited (Fig. 1B). At high concentrations of orthovanadate (1.25 and 0.625 mM), we observed some aspecific precipitates clearly unlike the staining normally seen in the absence of the PTPase inhibitor. These precipitates almost disappeared when lower concentrations (300, 150, and 75 µM) were added to the incubation medium (data not shown). Controls with P-ser or P-thr as a substrate or incubation without any substrate did not reveal any staining (Fig. 1C). Alkaline phosphatase was also negative. Acid phosphatase showed some staining of the stroma but not of the normal or malignant epithelium (data not shown).

Table 1. Patient and tumor characteristics versus histochemical staining in tissue of the patients and controls (patients MK and ML). The intensity of the staining was scored as (+)=weak, (++)=moderate, and (+++)=strong. M=male; F=female; Rx=previous radiotherapy; TNM=tumor classification system.

						staining	
Patient	Sex/Age	Grade	Rx	TNM	Site	Tumor	Non-tumor
BM	M/60	2	+	T2N0M0	larynx	++	+
CJ	M/58	2/3	-	T4N3M0	larynx	+/++	++
DA	F/48	2	+	T1N2M0	larynx	++	++
CY	M/77	2/3	+	T1N0M0	larynx	+++	+++
CU	M/67	2	+	T4N0M0	larynx	+++	++
DJ	M/78	3	+	T1N0M0	larynx	++	++
DK	M/41	2	+	T3N0M0	larynx	+++	++
DL	F/45	2	-	T2N1M0	tongue	++	+
DT	M/43	2	-	T2N0M0	tonsil	++	+
HX	F/56	2/3	-	T4N1M0	larynx	+++	++
MK	M/58	0	-	T0N0M0	palate	-	+
ML	M/58	0	-	T0N0M0	palate	-	+

Figure 1. Enzyme histochemical detection of PTPase activity. Malignant tissue and normal palate were subjected to an enzyme histochemical assay to detect protein tyrosine phosphatases activity, using phosphotyrosine (A, B, D) or phosphoserine (C) as a substrate. The reaction with phosphotyrosine was strongly inhibited by incubation of the substrate in the presence of 75 µM orthovanadate (B). Magnification: 100 x. White arrows: tumor (A,B); double black arrows: tumor (C); black arrows: hyperplastic mucosa (A,B,C) or normal mucosa (D).




C
D

5.4 Discussion

Phosphotyrosyl proteins are thought to mediate stimuli of various growth factors and oncogenes affecting cell proliferation. Their levels are regulated by protein tyrosine kinases and protein tyrosine phosphatases. Results from previous studies on PTPase activities in a biochemical assay [20,22] showed increased enzyme activities in tumor tissues as compared to non-tumorous epithelia. Remarkably, PTPase activities were also increased when comparing non-tumorous tissues of cancer patients with controls.

At first glance, the observation of increased PTPase activity is hard to reconcile with the hypothesis that aberrant phosphorylation causes oncogenic transformation through underexpression of a PTPase. However, it has also been reported that dephosphorylation of specific tyrosyl residues can activate some specific PTKs, as has been shown with the *src*-like kinases [24]. A recent study used human breast cell lines that had been transformed by the oncogene *neu*, which is known to express PTK activity; the results showed increased expression of two specific PTPases, LAR and PTP1B [23]. Furthermore, the level of LAR-PTPase was directly correlated with the level of *neu* expression in rat mammary cancer. This correlation has not been confirmed yet in human breast cancer [7].

There are also reports that describe other functions of PTPases. Treatment of normal rat kidney (NRK) cells with orthovanadate, which inhibits PTPase activity, not only increased the amount of phosphotyrosine in those cells but induced at the same time a transformed phenotype [8,14,15]. These findings presume a direct role of PTPases in cell differentiation. Receptor-like phosphatases also appear to be involved in contact inhibition [12] as density-arrested fibroblasts demonstrate an increased PTPase activity as compared to cells in the growth phase. At present, the exact role of PTPases in oncogenic transformation is not clear. It is known that PTPases constitute a large family of enzymes of which each member may have a distinct role in cell proliferation. Specific PTPases have been shown to be responsible for the activation of particular PTKs. Receptor-like PTPases appear to be involved in cell-cell interactions and may mediate contact inhibition of cells, while other PTPases counteract the PTKs in maintaining the balance between phosphorylation and dephosphorylation.

In the present study only slight differences were noted between the hyperplastic epithelium bordering the tumor and carcinoma tissue. Nonetheless, the differences between carcinoma and normal epithelium from control patients were significant. We could not detect any differences in staining intensity related to grade, stage, previous radiotherapy, or site. This was not surprising; in the biochemical assays, PTPase activity was not related to any of these clinicopathological parameters [22].

One remarkable finding was the strong reactivity of the surrounding connective tissue located close to the carcinoma cells. It has been suggested that this is the result of a mechanical compression of the stroma by the growing tumor. Otherwise, it might reflect the production of tumor factors by the neoplastic cells, which could be responsible for infiltrative growth and/or metastasis. However, collagen fibers constitute the major part of connective tissues, and are known to react aspecifically with lead salts [1]. A certain degree of diffusion of the reaction product, in this case lead phosphate, always occurs throughout the tissue sample. Therefore, the collagen fibers close to the tumor or epithelial cells are more likely to bind more lead salts than the collagens located at some distance from these cells. Indeed, with electron microscopy, we could confirm the aspecific binding of lead salts to collagen fibers (data not shown).

Since no staining in the controls with P-ser or P-thr as substrate was present, aspecific staining could be excluded. Furthermore, enzyme activity was strongly inhibited with orthovanadate, which is considered to be a specific PTPase inhibitor. No reaction was observed after incubation for alkaline and/or acid phosphatases. All these findings buttress the assumption that this method is specific for PTPases.

It is worth noting that the assay used in this experiment measures the total protein tyrosine phosphatase activity in the cells that react with an artificial substrate such as P-tyr. The enzyme activity quantitated by the present histochemical method is the sum of a number of specific and possibly non-specific enzyme activities related to dephosphorylation of phosphotyrosines. Consequently, variations in the activity of a specific PTPase may not be detected due to the wealth of other PTPases in the total PTPase activity. This makes inferences about the significance of increased PTPase activity in tumor tissue as compared with non-tumor tissue rather hazardous.

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CHAPTER 6

CLINICAL RELEVANCE OF PROTEIN TYROSINE (DE-) PHOSPHORYLATION IN HEAD AND NECK CANCER

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(submitted for publication)

Summary

Previous studies have shown that protein tyrosine (de-)phosphorylation plays an important role in head and neck cancer. Protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTPase) activities in the cytosol of tumor tissue were significantly increased compared to normal tissue of cancer patients as well as controls. Additionally, the enzyme activities in normal tissue of tumor patients were significantly higher than that in normal tissue of the control group.

In this paper we have correlated the cytosolic and membranous PTK- and PTPase activity of tumor and non-tumor tissue with several clinical and histological parameters which are known to influence the clinical outcome. Furthermore we have analyzed the value of the enzymes activities as an independent predictor of clinical behaviour and occurrence of second primary tumors.

We confirmed our earlier observations that cytosolic as well as membranous PTK-activities and cytosolic PTPase activities in tumor tissues are increased compared to non-tumor tissues and controls. Moreover, we also confirmed the findings of increased enzyme activities in non-tumor tissues compared to control tissues. This finding in, histologically-proven healthy mucosa is highly interesting as it indicates that these biochemical changes are obviously not (yet) translated into morphological changes. Significant differences were found in membranous PTK-activity when grouping the patients according to sex, tumor localization, lymph node metastasis and previous radiotherapy.

During the follow-up period, no relation could be found between enzyme activities in tumor and/or non-tumor tissues and disease-free interval or occurrence of second primary tumors.

6.1 Introduction

As improvements in cure of head and neck cancer have hardly been revolutionary in the last decades, a continued search for molecular prognostic parameters for head and neck squamous cell carcinoma is mandatory. Individual clinical outcome is hard to predict with the current classification, which is based on clinical and histological parameters [2]. If a molecular marker or a group of markers could predict the outcome of treatment of an individual patient, irrespective of the known clinical or histological parameters, we could change treatment modalities by making them more or less aggressive, depending on the predicted prognosis. Furthermore, these markers are needed to improve early detection of recurrence and/or second primary tumors. Finally, if a molecular marker appears to be correlated to clinical and/or histological parameters, this finding could give us more insight into the process of oncogenesis.

Research on molecular markers in tumors has been focused on oncogenes and more recently on tumor suppressor genes. Many oncogenes appear to code for protein tyrosine kinases (PTK), a group of enzymes which can phosphorylate proteins on tyrosyl residues. The state of phosphorylation of tyrosyl residues in proteins plays a crucial role in regulating cell growth and differentiation. The steady state level of tyrosine phosphorylation of cellular proteins depends on the interaction of PTKs and protein tyrosine phosphatases (PTPases). Both receptor (e.g. epidermal growth factor receptor, EGFr) and non-receptor (e.g. *src* family) PTKs have been identified and a number of tyrosine kinases appear to function as oncogenes [11]. As is the case for PTKs, PTPases constitute a family of intracellular and transmembrane proteins [8,27].
Alteration in the regulation of the phosphorylation state of tyrosyl residues on various cellular proteins appears to be an important pathway towards neoplastic transformation [4,25,28]. Further characterization of the role and regulation of these enzymes could provide clues to an understanding of the complex process of signal transduction during cell growth and differentiation.

Amplification or expression of mutated forms of oncogenes with PTK activity has been directly associated with neoplastic growth [28] and is sometimes related to the clinical behavior of certain tumors and thus to the prognosis of the patient [21,22,24]. The relationship between PTK activity and the growth and/or differentiation of human tumors has been described by several authors [7,9,30]. Less research has been done on the relation of PTPases to cancer [29].

Recently we reported on PTK activity and PTPase activity in head and neck cancer [29,30]. We observed an increase in activity of both enzymes in tumor tissue compared to normal tissue of cancer patients as well as to that of controls. Moreover, we found a significant increase in PTK activity in normal, non-tumorous mucosa of cancer patients compared to controls. This finding suggests an enzymologically 'premalignant' state of the non-tumorous mucosa of cancer patients. In Western blot experiments, we also found increased tyrosyl phosphorylation of endogenous proteins in tumors compared to normal tissue, both in cancer patients and in controls.

In this paper we correlate the PTK and the PTPase activity of tumor and non-tumor tissue with several clinical and histological parameters known to influence the clinical outcome. Furthermore we analyze the value of the activities of these enzymes as an independent predictor of clinical behavior, recurrence, and occurrence of second primary tumors.

6.2 Materials and Methods

6.2.1 Clinical material

Tumor tissue was obtained from 107 patients with squamous cell carcinoma of the upper aerodigestive tract. Tissue samples were taken from the surgical specimen by the pathologist immediately after resection. Samples were also obtained during staging diagnostic endoscopy procedures by dividing the diagnostic biopsies for purposes of this study. In 79 patients, samples were also taken from a histologically proven non-tumorous part of the mucosa. In addition, we obtained 31 control samples from patients without cancer who underwent elective surgery for other causes. All tissues were stored at minus 80°C until use in the diverse assays.

Histological diagnosis was performed according to WHO criteria [10]. Histology of previously untreated surgical specimens was scored according to the criteria proposed by Annoroth [1]. Tumor specifications (TNM classification, localization, and stage) were made according to UICC [10]. Sixty-one tumors were laryngeal; the others were from various head and neck localizations. Thirty-seven patients had lymph node metastasis; 15 of these patients had extracapsular tumor growth.

Patients' ages lay between 37 and 86 years (mean 62 ± 12 y. for the cancer patients and 47 ± 14 for the control patients). Twenty-one of the cancer patients and nine of the control patients were female. All but four patients were moderate to heavy smokers (more than 10 cigarettes a day), and 69 patients had an average daily

alcohol consumption of two or more glasses. Five patients had a synchronous HNSCC at the time of diagnosis or developed a second primary tumor during the follow-up. Six patients had previously been treated elsewhere for squamous cell carcinoma of the head and neck or lung and were currently being treated for a second primary. Twenty-one patients had been treated before by radiotherapy and/or surgery and were undergoing treatment for recurrent disease at the time of the study. These patients were not included in the disease-free interval and survival analysis. They were considered as a separate group when analyzing the different clinical parameters.

The follow-up period of patients still alive (n=75) ranged from 213 to 1187 days (median 761 days). Forty-eight patients developed recurrent disease. Death from disease-related causes occurred in 23 patients within two years, while four patients died after two years of follow-up. Three patients died of non-tumor-related disease within two years. Two patients with synchronous carcinomas of the larynx and the lungs were lost in the follow-up.

6.2.2 Sample preparation

At least 10 mg of tissue was necessary for the assays (usually between 30 and 60 mg was obtained). The samples were thawed and then lyzed with a mechanical homogenizer (Omni 1000™) in two volumes of extraction buffer. Samples used for the PTK assay were extracted in buffer containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 50 KIU/ml aprotinin. The suspensions were centrifuged for 10 min at 800 *g* in order to remove cell debris and nuclei. The supernatants were centrifuged during 60 min at 48,000 *g*. The resulting supernatants (referred to as 'cytosolic fraction') were used to determine the cytosolic PTK and PTPase activity. The remaining pellets were resuspended in 200 µl of a buffer solution containing 50 mM TRIS-HCl (pH 7.5), 20 mM Mg-acetate, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, 1 mM dithiothreitol to which 0.5% Nonidet P-40 was added. Then 30 mM Na_3VO_4 was added to the samples to be used in the PTK assay. After sonication (2 x 10 s) suspensions were centrifuged at 48,000 *g* for 60 min. The resulting supernatants (the solubilized membrane fraction, referred to as 'membrane fraction') were used to determine the membrane-bound PTK and PTPase activity.

6.2.3 Determination of the protein concentration

The protein content of cytosolic and membrane fractions was determined according to the method of Lowry [15]. A bovine serum albumin solution diluted in water was

used as a standard. If contamination of the fractions with blood was suspected, a correction of the protein content was made by spectrophotometric determination of the hemoglobin concentration in the fractions. We assumed that the PTK and PTPase activity of red blood cells was non-significant [7].
Due to the limited sample size of some biopsies, the amount of protein for certain samples was too low to perform all assays. Therefore, the number of samples analyzed for each enzyme is less than the total number of patients (n=107).

6.2.4 Determination of protein tyrosine kinase activity

The PTK activity of the fractions was determined by a non-radioactive dot-blot assay as described by Rijksen et al. [19]. This method is based on the detection of phosphorylated tyrosine residues in an artificial substrate known as poly (glutamic acid;tyrosine 4:1) (PGT, Sigma, St.Louis, MO) by a monoclonal antibody to phosphotyrosine (IG2, Oncogene Science, Manhasset, NY, USA). All samples were phosphorylated in duplicate, and detected in duplicate resulting in four values for each sample. Variance was less than 10% for each sample. The PTK activity is expressed as picomoles tyrosyl residues which are phosphorylated in PGT per minute per milligram of sample protein (=pmol P-tyr/min.mg protein).

6.2.5 Determination of protein tyrosine phosphatase activity

For the determination of PTPase activity a radiolabeled PTPase substrate had to be prepared. PGT (5 mg) was phosphorylated in vitro by PTKs extracted from A431 cells (1 mg protein) in a buffer containing 50 mM HEPES (pH=7.2), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM NaF, 1 mM EDTA/EGTA, 1 mM dithiothreitol, 100 µM orthovanadate, and 100 µM ^{33}P-ATP with a specific activity of 0.15 Ci/mmol for 20 h at 20°C. PGT was precipitated in TCA. The precipitate was collected by centrifugation and washed with ice-cold TCA to remove unincorporated ^{33}P-ATP. The final precipitate was resolved in 1 M NaOH. A buffer change was accomplished by passing the phosphorylated PGT over a Sephadex G-25 PD 10 column (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM HEPES pH 7.5, containing 0.1 mM EDTA and 1 mM dithiothreitol. The substrate fraction was eluted in 2 ml. Then ^{33}P-labelled PGT was used as substrate for PTPases at a concentration of 0.25 mg/ml and a ^{33}P-phosphate incorporation of 5 mmol per mol tyrosine.
Dephosphorylation of ^{33}P-labeled PGT was carried out at 37°C in a total volume of 40 µl containing 50 mM HEPES, 0.1 mM EDTA, and 1 mM dithiothreitol. Initially, the assay on the cytosolic fraction was performed at pH 7.0 in 50 patients. The other cytosolic fractions and all of the membranous fractions were assayed at pH 7.5. Conditions were changed to a slightly different pH to optimize the assay. The

pH curve showed no indications of the presence of PTPases with different pH optima (data not shown).

The reaction was started by adding the sample (2 µg/assay). After 10 min the reaction was stopped by the addition of 200 µl 12% TCA. Radiolabeled PGT was precipitated. After centrifugation at 14,000 *g* for 10 min, the amount of liberated ^{33}P-PO_4 in the supernatant was quantified by liquid scintillation counting. PTPase activity is expressed as pmol PO_4/min.mg protein.

6.2.6 Statistical methods

To evaluate differences in PTK and PTPase activity in tumor samples, non-tumor samples and control samples, analysis of variance was applied as appropriate. To evaluate the relations between the enzymological and clinicopathological parameters, a multiple regression analysis was performed. Cox's proportional hazard regression analysis and log rank tests were used to evaluate the disease-free interval and the survival in the whole patient group as well as in several subgroups. PTPase activities determined at pH 7.0 were recomputed to PTPase activity at pH 7.5 by a monotonic, linear transformation, making use of the linear relation in both pH groups between the experimental values and their expected normal deviations. The specificity of the PTPase assay remained unchanged under the slightly different pH conditions (data not shown). The SPSS (SPSS Inc., Chicago, IL, USA) and NCSS (Dr. Jerry L. Hintze, Kaysville, UT, USA) statistical software programs were used throughout.

6.3 Results

6.3.1 Enzyme activities

Mean PTK and PTPase activities of the cytosolic and membranous fractions of tumorous, non-tumorous (from normal mucosa of patients with head and neck cancer) and control samples are presented in Table 1.

The mean value of cytosolic and membranous PTK activity of tumor samples is 4.1, respectively 3.2 times higher compared to that of normal mucosa of the control group ($p < 0.001$). In paired t-tests, the mean value of cytosolic and membranous PTK activity of tumor samples is 2.2, respectively 1.7 times higher compared to that of the non-tumorous mucosa of the same patients ($p < 0.001$). Non-tumorous mucosa of patients with head and neck cancer showed a 1.9-fold increase in mean cytosolic and membranous PTK activity compared to normal mucosa of the control group ($p < 0.001$, respectively $p = 0.004$).

Table 1. Cytosolic and membranous PTK and PTPase activities in tumorous, non-tumorous, and control mucosa. The values are mean ± SD, with the number of samples in parentheses. PTK and PTPase activities are expressed in pmol P-tyr/mg protein.min. Cytosolic PTPase activities are represented according to the two different assay conditions: pH 7.0 and pH 7.5 respectively (see Materials and Methods).

	Tumor	Non-tumor	Control
Cytosol PTK (*n*)	252 ± 150 (104)	117 ± 91 (80)	61 ± 39 (31)
Membranes PTK (*n*)	3111 ± 2476 (94)	1870 ± 2059 (70)	983 ± 739 (18)
Cytosol PTPase (pH 7.0) (*n*)	425 ± 214 (50)	309 ± 208 (37)	238 ± 106 (11)
Cytosol PTPase (pH 7.5) (*n*)	603 ± 238 (57)	361 ± 206 (44)	230 ± 166 (20)
Membranes PTPase (*n*)	993 ± 580 (37)	612 ± 507 (30)	764 ± 514 (18)

The mean value of cytosolic and membranous PTPase activity (at pH 7.5) of tumor samples is 2.6, respectively 1.3 times higher compared to that of normal mucosa of the control group ($p < 0.001$, respectively $p = 0.146$). In paired t-tests, the mean value of cytosolic and membranous PTPase activity of tumor samples is 1.7, respectively 1.6 times higher compared to that of the non-tumorous mucosa of the same patients ($p = 0.001$, respectively $p < 0.001$).

Non-tumorous mucosa of patients with head and neck cancer showed a 1.9-fold increase in mean cytosolic PTPase activity compared to normal mucosa of the control group ($p = 0.010$). No significant differences could be observed in the membranous PTPase activity of non-tumor tissue and controls.

Essentially the same significant ratios in cytosolic PTPase activity of tumor, non-tumor and control samples could be observed at pH 7.0.

6.3.2 Relations with other parameters

In order to evaluate the influence of tobacco and alcohol consumption on the enzyme activities, we analyzed these variables in cancer patients. No relation could be found between total tobacco consumption and enzyme activities in either tumor or non-tumor tissues. However, only four patients were non-smokers, which makes statistical significance unlikely. In the patient group, 38 persons consumed less then two glasses of an alcoholic beverage a day and were thus considered as social drinkers. No differences in enzyme activities in tumor or in non-tumor tissues could be found between the social drinkers and the heavy drinkers. The age of the patients had no influence on enzyme activities.

Apart from the membranous PTPase activity, all other enzyme activities of non-tumor tissue were significantly higher in women as compared to men. In tumor tissue, a significant increase was found in the membranous PTK activity of female patients ($p=0.002$). In control tissue, these sex differences were not found.

Considering the localization of the tumors, we observed that the membranous PTK activity of tumor tissue from laryngeal cancer was significantly lower than from other tumor sites ($p=0.006$).

Patients with tumor-positive lymph nodes had higher cytosolic and membranous PTK activity in their tumor tissue than those patients with no lymph node involvement (respectively $p=0.004$ and $p=0.029$). However, patients with extracapsular tumor growth demonstrated no different enzyme activities than the other patients with tumor-positive lymph nodes. Tumor size and/or stage had no influence on enzyme activities.

Enzyme activities were also related to the seven histological parameters proposed by Annoroth [1]. Enzyme activities did not show appreciable differences between classes of histological parameters. Patients who had been treated previously by radiotherapy turned out to have lower membranous PTK activities than previously untreated patients ($p=0.0006$).

In a multivariate analysis, sex and previous treatment remained the only two independent factors.

Some relevant relations between enzyme activities and clinical parameters are shown in Table 2.

Table 2. Relation of cytosolic and membranous PTK and PTPase in tumor tissues with several clinical prognostic parameters. PTPase activities were determined at pH 7.5 (see Materials and Methods).

Sex	Male	(*n*)	Female	(*n*)	p =
PTK cytosol	246 ± 152	(84)	280 ± 142	(20)	0.346
PTK membrane	2790 ± 2179	(80)	4943 ± 3281	(14)	**0.0023**
PTPase cytosol	578 ± 243	(41)	669 ± 219	(16)	0.196
PTPase membrane	937 ± 494	(26)	1124 ± 757	(11)	0.377
Previous treatment	Untreated	(*n*)	Previous radiotherapy	(*n*)	p =
PTK cytosol	261 ± 151	(83)	219 ± 144	(21)	0.249
PTK membrane	3572 ± 2471	(73)	1509 ± 1752	(21)	**0.0006**
PTPase cytosol	611 ± 243	(50)	548 ± 203	(7)	0.523
PTPase membrane	961 ± 569	(33)	1256 ± 687	(4)	0.344
Localization	Larynx	(*n*)	Non-larynx	(*n*)	p =
PTK cytosol	237 ± 141	(63)	276 ± 162	(41)	0.190
PTK membrane	2555 ± 2493	(57)	3968 ± 2220	(37)	**0.0062**
PTPase cytosol	539 ± 258	(19)	636 ± 224	(38)	0.149
PTPase membrane	827 ± 601	(10)	1054 ± 571	(27)	0.297
Tumor size	T1 + T2	(*n*)	T3 + T4	(*n*)	p =
PTK cytosol	243 ± 145	(39)	268 ± 161	(44)	0.456
PTK membrane	3317 ± 2327	(35)	3616 ± 2630	(38)	0.598
PTPase cytosol	541 ± 235	(22)	662 ± 234	(29)	0.073
PTPase membrane	810 ± 421	(14)	1126 ± 650	(19)	0.117
Lymph node metastases	Negative	(*n*)	Positive	(*n*)	p =
PTK cytosol	222 ± 133	(68)	309 ± 164	(36)	**0.0043**
PTK membrane	2722 ± 2390	(63)	3902 ± 2497	(31)	**0.0291**
PTPase cytosol	595 ± 227	(26)	613 ± 255	(31)	0.786
PTPase membrane	902 ± 479	(20)	1099 ± 679	(17)	0.310

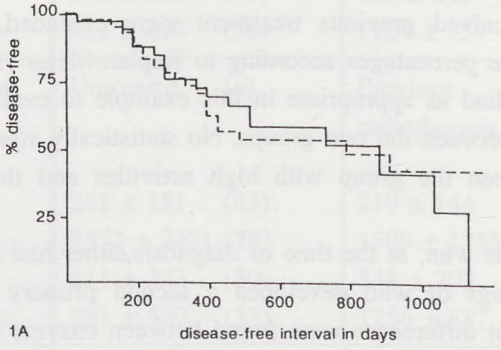
During the follow-up period, no relation could be found between enzyme activities in tumor and/or non-tumor tissues and disease-free interval using Cox's analysis. Subdifferentiation of the patient group according to localization, lymph node status, and/or sex did not reveal a prognostic value for the various enzyme activities either. An illustration is given in Figure 1. Patients were divided into two groups: patients with enzyme activities higher than the median value and those with activities lower than the median value. This differentiation was made for cytosolic and membranous PTK activity (Fig. 1A and 1B) as well as for cytosolic PTPase activity (Fig. 1C). Patients who had received previous treatment were excluded. A product limit estimate of disease-free percentages according to Kaplan-Meier [12] was applied. A log rank test was applied as appropriate in this example to evaluate differences in disease-free survival between the two groups. No statistically significant differences could be found between the group with high activities and the group with low activities.

In the group of patients who, at the time of diagnosis,either had two carcinomas of the UADT or the lungs or who developed a second primary tumor during the follow-up period, slight differences were found between enzyme activities of tumor and/or non-tumor tissues compared to the rest of the tumor patients. However, as the group was small (n=5), statistical analysis is unlikely to support this finding. The mean values for enzyme activities are listed in Table 3.

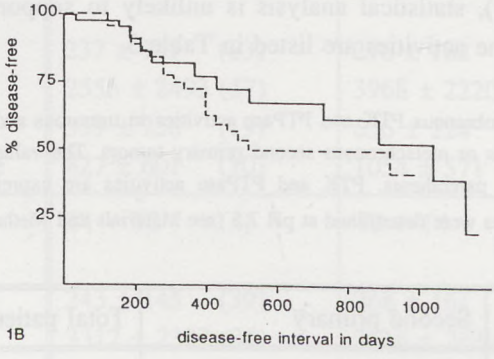
Table 3. Cytosolic and membranous PTK and PTPase activities in tumorous and non-tumorous mucosa of patients with synchronous or metachronous second primary tumors. The values are mean ± SD with the number of samples in parentheses. PTK and PTPase activities are expressed in pmol P-tyr/mg protein.min. PTPase activities were determined at pH 7.5 (see Materials and Methods).

Enzyme activities	Second primary		Total patient group	
Tumor tissue				
PTK cytosol	306 ± 207	(n=5)	252 ± 150	(n=104)
PTK membrane	1910 ± 1092	(n=5)	3111 ± 2476	(n=94)
PTPase cytosol	723 ± 183	(n=5)	603 ± 238	(n=57)
Non-tumor tissue				
PTK cytosol	102 ± 77	(n=4)	117 ± 91	(n=91)
PTK membrane	1573 ± 1489	(n=3)	1870 ± 2059	(n=70)
PTPase cytosol	394 ± 270	(n=4)	361 ± 206	(n=44)

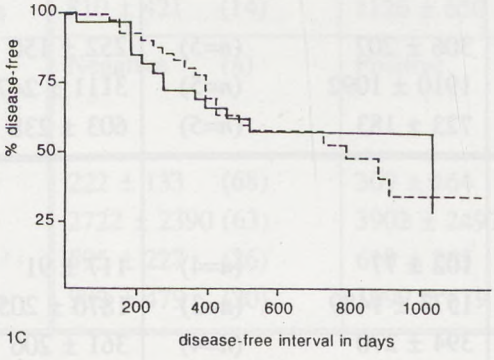
Figure 1. Disease-free interval plots. On the vertical axis the percentage of patients with no signs of recurrent disease. On the horizontal axis the disease-free interval in days. The lines represent those patients with an enzyme activity higher (------) or lower (_____) than the median value. Figure 1A represents the disease-free interval plot for the cytosolic PTK activity, 1B for the membranous PTK activity and 1C for the cytosolic PTPase activity. Membranous PTPase activity is not shown due to the limited size of the group. Differences between the several groups with higher activities compared to those with lower activities are not significant.



1A



1B



1C

6.3.3 Mutual correlations between each of the enzyme activities

Apart from the relations with the clinical and histological parameters, mutual correlations between each of the measured enzyme activities were investigated. In control tissues, a correlation was found between the cytosolic and membranous PTPase activity ($r=0.85$, $p<0.001$). In the cancer patients, no correlation between the cytosolic and membranous PTK or PTPase activity was found (correlation coefficient $r<0.5$). Nor did we find correlations in enzyme activities between tumor and non-tumor tissues.

6.3.4 Ratios of enzyme activities

In an earlier report we showed that the ratio of tumor and non-tumor PTK activities (T/N ratio) in the same patient was associated with previous radiotherapeutic treatment. In this series, a decrease in the T/N ratio of cytosolic PTK activity was seen in those patients who had previously undergone radiotherapy compared to untreated patients (2.90 ± 0.59 versus 4.06 ± 0.86). However, this decrease was not statistically significant ($p=0.2699$). No differences were present in the T/N ratio of membrane-bound PTK activity (3.19 ± 1.15 versus 3.92 ± 0.79, $p=0.605$).

6.4 Discussion

Increasing evidence suggests that malignant transformation requires changes in the activity of both oncogenes and tumor suppressor genes [5,14,16,26,31]. Oncogenes activated by a variety of mechanisms frequently have been shown to code for growth factors, receptor and non-receptor tyrosine kinases, and other proteins involved in signal transduction. Increased or altered expression of oncogenes during malignant transformation would be expected to change the specific or total PTK activity in tumor cells.

Indeed, in previous reports, increased PTK activities have been found in several tumors such as breast, brain, and head and neck cancer [7,9,18,29,30].

For the maintenance of controlled cell proliferation, the balance between tyrosine kinase and tyrosine phosphatase should remain undisturbed. Elevation of the content of tyrosine-phosphorylated proteins has been frequently found in transformed or malignant cells, indicating a disturbance of the balance between tyrosine kinases and tyrosine phosphatases. It seems quite likely that, besides changes in PTK activity, alteration of PTPase activity can also influence the amount of tyrosine-phosphorylated proteins.

At present, it is not known whether tumor suppressor genes are involved in phosphorylation or dephosphorylation of the proteins that play a role in signal transduction.

In the present study, the tyrosine kinase and tyrosine phosphatase activity of tumor tissue and non-tumor tissue of patients with head and neck cancer were determined and compared with enzyme activities in control tissues. Additionally, we examined whether the enzyme activities could have a prognostic significance in the recurrence of the disease or in the occurrence of a second primary tumor. Furthermore, we examined the relations between the enzyme activities and several clinical and histological parameters.
We confirmed our earlier observations that cytosolic as well as membranous PTK activities and cytosolic PTPase activities in tumor tissues are increased compared to non-tumor tissues and controls. Moreover, we also confirmed the findings of increased enzyme activities in non-tumor tissues compared to control tissues.

This finding in mucosa that is histologically proven to be healthy is highly interesting. It indicates that these biochemical changes are obviously not (yet) translated into morphological changes. The increase in enzyme activities is highly unlikely to be an effect of tobacco and/or alcohol consumption, since we did not find any relation between those activities and the carcinogens in either cancer patients or controls [30]. Another explanation could be an enzymological premalignant state of the non-tumor mucosa. Therefore, this could be a predictor of a second primary tumor. Unfortunately, we were not able to find any significant differences in enzyme activities in patients who developed a second primary compared to those who did not. However, this patient group is small, and the follow-up of the whole patient group is still relatively short (median 25.4 months). Thus, definitive conclusions about the predictive value of increased enzyme activities in non-tumor mucosa can only be drawn after several years.

In order to assess the value of enzyme activity determination as a predictor for clinical behavior, we analyzed the survival and disease-free interval of the patients. We could not find a relation to any of the enzyme activities measured. We do realize that the follow-up is still short. Yet as the survival and disease-free curves are nearly synchronous, we do not expect a longer follow-up to reveal significant differences. We therefore assume that the measurement of the activities of these enzymes is not meaningful in predicting clinical outcome.

In an earlier report on breast cancer, a relation was found between cytosolic PTK activity and tumor relapse [9]. As the total cytosolic PTK activity is the sum of the activities of several PTKs we must assume that the role and contribution of the diverse PTKs in head and neck cancer is different from that in breast cancer. However, we also found some indications that similarities do exist between the two types of cancer. In both types of tumors, a significant increase was found in cytosolic PTK activity in node-positive patients compared to those without lymph nodes involvement. Thus, we may conclude that cytosolic PTK activity is involved in or associated with metastases. Previous studies have shown that ± 70% of the PTK activity in breast cancer could be attributed to the presence of *c-src*. At present, it is not known whether this is also the case in head and neck cancer. Membranous PTK activity was also increased in node-positive tumors. As epidermal growth factor receptor (EGFr) is frequently expressed in head and neck squamous cell carcinoma [3,6,13,17,20,23,24], this membrane-bound receptor could be the origin of (a part) of the membranous PTK activity. However, there are more growth factor receptors with PTK activity, including the oncogene *c-erbB2/neu*, which have also been demonstrated in head and neck cancer. Nevertheless, it is tempting to speculate that *c-src* and growth factor receptors are implicated in the process of metastasis of cancers which express increased cytosolic and membranous PTK activity in node-positive tumors.

Remarkably, membranous PTK activity was lower in previously treated patients. All these patients had previously received radiotherapy before the enzyme activities in the recurrent tumor were analyzed. Obviously, radiotherapy has an effect on receptor tyrosine kinases. It may be a selecting effect of radiotherapy in the sense that cells with low membranous PTK activity could be more radioresistant than other cells. Therefore, surviving cell clones would demonstrate lower membranous PTK activity. It cannot be an effect of increased fibrosis and the concomitant relative decrease in cell proteins compared to collagens, as the latter are eliminated during centrifugation. Moreover, if the influence of fibrosis was indeed responsible for the differences in membranous PTK activity, all enzymes would show a decrease, even in the non-tumor samples. And that was not the case.

Another striking finding was that, apart from the membranous PTPase activity, all enzyme activities of non-tumor tissue were significantly higher in women compared as to men (data not shown). In tumor tissue, a significant increase was also found in the membranous PTK activity of female patients. The latter finding could be explained by the fact that in this series, women had more non-laryngeal cancer with

lymph node metastases than men. We showed that these variables are associated with increased membranous PTK activity. Concerning the increases in enzyme activities in female non-tumor tissue as compared to men, it is intriguing that we did not observe these sex differences in control mucosa. We are unaware of reports of a higher incidence of second primaries in women. As we do not know whether an increase in enzyme activities in non-tumor tissue forms a predictor of a second primary, we will have to closely monitor the women in this patient group in order to confirm or reject this supposition.
Multiple regression analysis revealed that localization and lymph node metastases were not independent variables but were associated with sex and previous radiotherapy. This was not surprising, considering the heterogeneous distribution in this series.

Concluding, we can affirm that while enzyme activities are not direct prognostic factors for disease-free interval or occurrence of second primaries, they are clearly correlated with several relevant clinical parameters.
Apart from the results from tumor tissue, the intriguing finding of increased enzyme activities in non-tumor mucosa of cancer patients warrants further research on signal transduction by tyrosine (de-)phosphorylation in malignancy.

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CHAPTER 7

PROTEIN TYROSINE KINASE INHIBITORS AFFECT CELL-GROWTH AND PROTEIN TYROSINE KINASE ACTIVITY IN A HEAD AND NECK SQUAMOUS CELL CARCINOMA CELL LINE

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Summary

Phosphorylation of proteins on tyrosine residues by protein tyrosine kinases is a key mechanism in signal transduction of the normal and malignant cell. We questioned whether the inhibition of PTK activity could influence the growth of a head and neck squamous cell carcinoma cell line.

To investigate this, we used cultured cells derived from human head and neck cancer and inhibited them with several known PTK inhibitors. The outcome revealed how PTK inhibition altered the cell growth. We also evaluated how PTK activities and levels of tyrosyl phosphorylation in the cell lines were influenced by PTK inhibitors.

Herbimycin A and tyrphostin RG 50875, showed cell-growth inhibition. At least for herbimycin A the inhibition was dose-dependent. Two other PTK inhibitors, tyrphostin RG 13202 and genistein, had no effect on cell growth. Herbimycin A, that specifically inhibits *src*-like tyrosine kinases, also had a dose-dependent influence on the phosphotyrosine levels in the cells. The growth-inhibiting RG 50875 as well as the non-inhibiting RG 13202 and genistein had no effect on the phosphotyrosine levels in the cells. No changes in phosphotyrosine protein patterns were seen in any of the inhibitors.

After pre-incubation of the cells with the various inhibitors, a marked increase in both cytosolic and membranous PTK activity was measured instead of the expected decrease in kinase activity. An explanation for this phenomenon is suggested.

Concluding, cell growth inhibition in a head and neck squamous cell carcinoma cell line can be achieved with PTK inhibitors. This implies that PTK inhibitors are potential anti-proliferative drugs in head and neck cancer. The fact that herbimycin A is capable of inhibiting cell growth and reducing P-tyr levels is an indication that *src*-like tyrosine kinases play a role in the proliferation of this cell line.

7.1 Introduction

Phosphorylation of proteins on tyrosine residues is a key biochemical reaction in signal transduction [4,12,23,24,42,44]. In several tumors increased protein tyrosine kinase (PTK) activity was found; in some tumors, PTK activity was correlated with histology [16,29], lymph node metastasis [29,30,47], or prognosis [11,16,29]. Increased levels of phosphorylated tyrosyl residues in tumors compared to non-tumorous tissues have also been reported [36,45]. In many cases, the enhanced PTK activity originating from the (over)expression of oncogene products or growth factor receptors was found to be essential to their transforming activity [2,32,39,44]. In head and neck cancer, we found increased PTK activity in tumor tissues compared to non-tumor tissues. The membranous PTK activity was related to sex, localization, lymph node metastasis, and previous treatment. We also found increased PTK activity in, histologically proven, non-tumor mucosa of cancer patients compared to controls [45-47].

Protein tyrosine kinases can be divided into two categories: (membrane-bound) receptor PTKs and non-receptor PTKs. Most of the receptor PTKs are growth factor receptors, such as epidermal growth factor receptor (EGFr) and platelet-derived growth factor receptor (PDGFr). Binding of specific ligand (growth factor) to the extracellular domain of these receptors results in an activation of the intracellular PTK activity, which precedes a cascade of reactions further downstream [31,39,44]. The best-known example of non-receptor PTKs is formed by the (proto-)oncogenes of the *src*-family. *Src* products like $pp60^{c\text{-}src}$ are involved in signal transduction. They are associated with other components, like activated growth factor receptors. $Pp60^{c\text{-}src}$ can be activated by dephosphorylation of a specific tyrosyl residue (tyr^{530}) [9,18]. Recently, *c-src* specific kinase (CSK), which is ubiquitously expressed, has been identified as a tyrosine kinase able to phosphorylate $pp60^{c\text{-}src}$. When raising the amount of CSK during an in vitro experiment, the level of phosphorylation of tyr^{530} increased. At the same time, a decrease in $pp60^{c\text{-}src}$ PTK activity was observed [26,52].

Protein tyrosine phosphatases are responsible for the dephosphorylation of phosphorylated proteins (reviewed in [42]). Therefore, PTPases are believed to counteract the overall PTK activity. However, specific PTPases can also be responsible for the activation of particular PTKs by dephosphorylation of tyrosyl residues (like tyr^{530} in $pp60^{c\text{-}src}$) [9,18]. As is the case for PTKs, PTPases constitute a family of intracellular and transmembrane proteins [12,42].

The exact mechanism of phosphorylation and dephosphorylation is complex and has not been completely elucidated. Presumably, certain protein tyrosine kinases can be (de-)activated by specific PTKs and/or specific PTPases.

In head and neck squamous cell carcinoma, an increased expression of EGFr has frequently been reported [1,6-8,10,19,20,25,35,37,38,41,50]. In several tumors, products of the *src*-family appear to be responsible for most PTK activity [5,30,48]. Therefore, inhibition of EGFr kinase activity or *src* kinase activity could have an influence on the proliferation of the cells.

In this paper, we explore the effect of several PTK inhibitors in cultured squamous cells derived from human head and neck squamous cell carcinomas. We examine how PTK inhibition alters cell-growth. We also try to correlate these findings with the effects of PTK inhibitors on the levels and patterns of phosphorylated proteins and PTK activities.

7.2 Materials and Methods

7.2.1 Cell culture

A head and neck squamous cell carcinoma (HNSCC) cell line was established at the Laboratory of Cancer Research at the University of Michigan (USA) and was generously donated by Dr. T.E. Carey (University of Michigan). It was derived from a well-differentiated squamous cell carcinoma of the lower alveolus (SCC-2). Other characteristics of the cell line are described elsewhere [15].

The cells were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1% $NaHCO_3$, 2 mM pyruvate, and 100 µg/ml gentamycin in a 5% humidified CO_2 atmosphere at 37°C. Cells were plated in 96-well plates (for cell-growth inhibition experiments) or in 56 cm^2 culture dishes (for the biochemical assays) (Nunc, Inc., Naperville, IL, USA).

7.2.2 Protein tyrosine kinase inhibitors

Four protein tyrosine kinase inhibitors were used in this study. Herbimycin A (HMA) is an antibiotic from the benzoquinoid ansamycin group isolated from Streptomyces hygroscopicus. It is described as an inhibitor of *src*-like kinases [13,14,17,43]. Tyrphostins RG 50875 and RG 13022 are considered to selectively inhibit the kinase activity of the EGF receptor in vitro; they also inhibit EGF-stimulated cell proliferation [22,49,51]. Compound RG 13022 is more stable in

aqueous solutions in comparison to RG 50875, which is the tyrphostin most frequently used in experiments. Therefore RG 13022 seemed the right choice for application in cell culture and in vivo. The isoflavone genistein is generally used as a PTK inhibitor with rather broad specificity [36]. Stock solutions of the compounds were made in 100% DMSO and diluted with the culture medium before use. Equivalent concentrations of DMSO served as vehicle controls and had no effect on cell-growth. Herbimycin A, genistein, and RG 50875 were purchased from Gibco (Gaithersburg, MD, USA). RG 13022 was a generous gift of Rhône-Poulenc Rorer Central Research (Horsham, PA, USA).

7.2.3 Cell-growth inhibition

For the dose-response studies, 4.0 x 10^3 cells/well were plated in 96-well plates. After 24 hours, culture medium was replaced with the same medium to which specific PTK inhibitors were added in increasing concentrations: herbimycin A (125-1000 ng/ml), RG 50875 (2-20 µg/ml), RG 13022 (2-20 µg/ml), or genistein (2-20 µg/ml). After 72 hours (unless otherwise indicated), cell-growth was quantified using a methylene blue staining technique, as described by Oliver [28].
Cell toxicity was evaluated by counting the loose cells in the medium after culturing for 72 hours in the presence of inhibitors. It appeared to be negligible under all conditions.

7.2.4 Immunoblotting

Cells (7 x 10^5) were plated in 56 cm^2 culture dishes. After 72 hours, culture medium was replaced with the same medium to which specific PTK inhibitors were added in the following concentrations: herbimycin A (125, 250, 400, or 625 ng/ml), RG 50875 (15 µg/ml), RG 13022 (15 µg/ml), or genistein (15 µg/ml). Thirty min before harvesting the cells, 100 µM vanadate was added to the dishes. After 24 hours of culturing in the presence of inhibitors, cells were washed trice with ice-cold phosphate-buffered saline solution containing 100 µM vanadate. The cells were then harvested by scraping in 120 µl extraction buffer, which contained 20 mM HEPES at pH 7.2, 20 mM Mg-acetate, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, glycerol 10%, 1 mM vanadate, 1 µm PMSF, 50 KIU/ml aprotinin, and 0.5% Nonidet P-40. The samples were sonicated twice for 5 s, solubilized for 30 min and centrifugated for 45 min at 48,000 x *g*. The resulting supernatant is referred to as the 'total cell lysate'. Protein was determined according to Bradford [3]. SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described elsewhere [45]. After boiling samples for 5 min, SDS-PAGE was carried out on a 10% separating gel. After electrophoresis [21], proteins were transferred electrophoretically to a

polyvinylidene difluoride membrane and blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline. Washing and further incubation was performed with 0.1% BSA in phosphate-buffered saline. Membranes were probed with monoclonal mouse antibody directed against phosphorylated tyrosine (P-tyr) (PY20, ICN Biomedicals, Costa Mesa, CA, USA). Immune complexes were visualized by using an immunogold-silver-staining method [33].

7.2.5 Enzyme assays

Cells (7 x 10^5) were plated in 56 cm^2 culture dishes. After 72 hours, culture medium was replaced with medium to which specific PTK inhibitors were added, and cells were cultured for another 24 hours. PTK inhibitors included herbimycin A (125, 250, or 625 ng/ml), RG 50875 (15 µg/ml), RG 13022 (15 µg/ml), or genistein (15 µg/ml). Cells were washed trice with ice-cold phosphate-buffered saline solution containing 7.44 mM EDTA. Then they were scraped in extraction buffer, which contained 20 mM HEPES pH 7.2, 1 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM PMSF, and 50 KIU/ml aprotinin. The samples were sonicated twice for 5 s, and the samples were clarified by centrifugation at 48,000 x *g* for 60 min. The resulting supernatant was used as the cytosolic fraction. The remaining pellet was solubilized in a buffer containing 20 mM HEPES at pH 7.2, 20 mM Mg-acetate, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, and 0.5% Nonidet P-40. This fraction was kept on ice for 1 hour, during which time it was sonicated twice for 10 s. The solubilized membrane fraction was obtained by clarification at 48,000 x *g* for 60 min. Protein was determined according to the method described by Bradford [3].

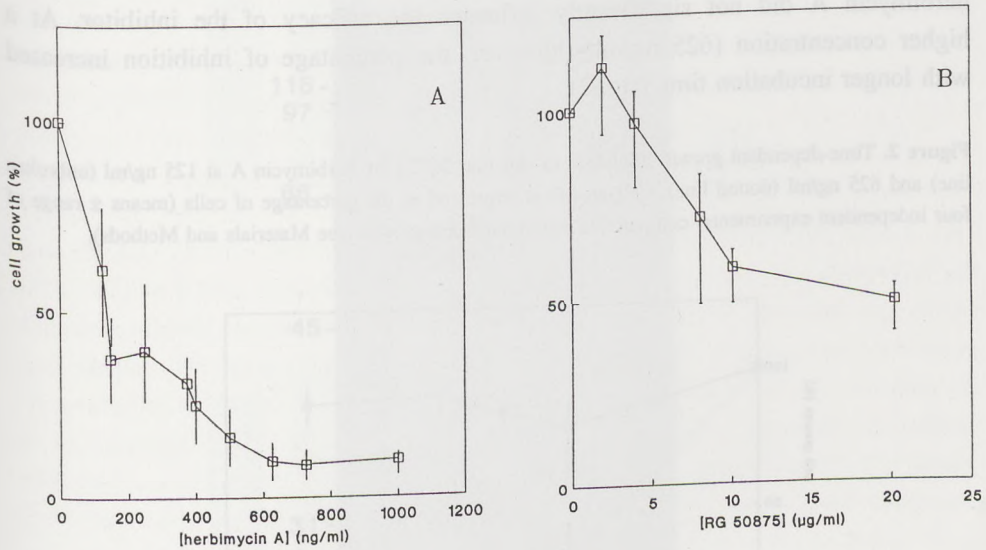
Protein tyrosine kinase (PTK) activities of the fractions were determined by an enzyme-linked immunosorbent assay (ELISA), as described earlier [34,40]. This method is based on the detection of phosphorylated tyrosine residues in an artificial substrate using poly (glutamic acid:tyrosine, 4:1)(PGT, Sigma, St. Louis, MO, USA) coated to the wells of a 96-well microtiter plate. After incubation with the samples and ATP, the number of phosphorylated tyrosyl residues was quantitated with a monoclonal antibody to phosphotyrosine (IG2, Oncogene Science, Manhasset, NY, USA) and a secondary peroxidase-labeled antibody. The optical density of the reaction was measured with an ELISA reader. Enzyme activities were compared to a rat spleen standard with known specific activity and expressed in pmol P-tyr/mg protein.min.

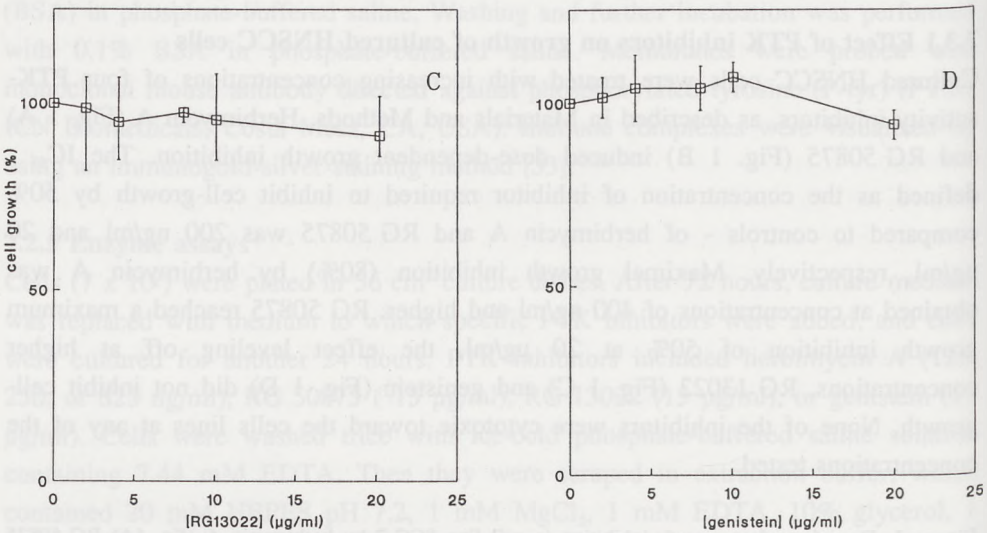
7.3 Results

7.3.1 Effect of PTK inhibitors on growth of cultured HNSCC cells

Cultured HNSCC cells were treated with increasing concentrations of four PTK-activity inhibitors, as described in Materials and Methods. Herbimycin A (Fig. 1 A) and RG 50875 (Fig. 1 B) induced dose-dependent growth inhibition. The IC_{50} - defined as the concentration of inhibitor required to inhibit cell-growth by 50% compared to controls - of herbimycin A and RG 50875 was 200 ng/ml and 20 µg/ml, respectively. Maximal growth inhibition (80%) by herbimycin A was obtained at concentrations of 400 ng/ml and higher. RG 50875 reached a maximum growth inhibition of 50% at 20 µg/ml, the effect leveling off at higher concentrations. RG 13022 (Fig. 1 C) and genistein (Fig. 1 D) did not inhibit cell-growth. None of the inhibitors were cytotoxic toward the cells lines at any of the concentrations tested.

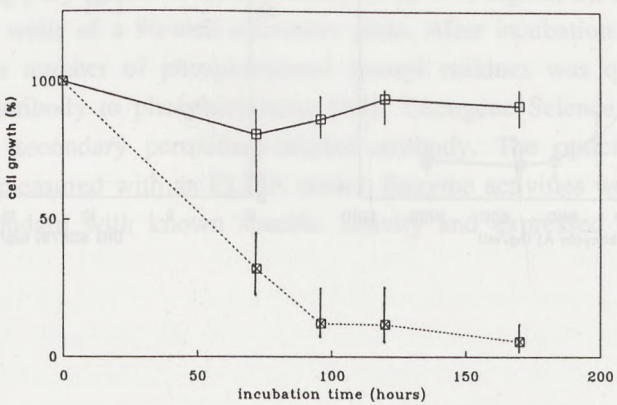
Figure 1. Dose-dependent growth inhibition in cell line SCC-2 by herbimycin A (fig. 1A) RG 50875 (fig. 1B), RG 13022 (fig. 1C) and genistein (fig. 1D). Cell-growth is expressed as the percentage of cells (means ± range of four independent experiments) compared to uninhibited cell-growth (see Materials and Methods).





Increasing the incubation times at suboptimal concentrations (125 ng/ml) of herbimycin A did not significantly influence the efficacy of the inhibitor. At a higher concentration (625 ng/ml), however, the percentage of inhibition increased with longer incubation time (fig. 2).

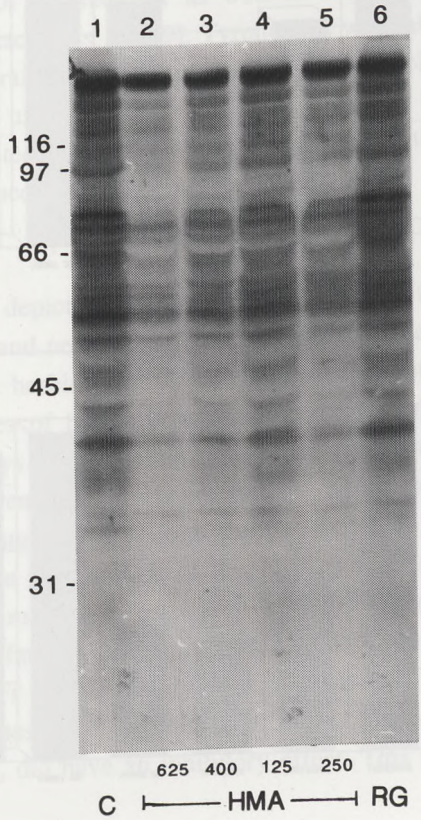
Figure 2. Time-dependent growth inhibition in cell line SCC-2 by herbimycin A at 125 ng/ml (unbroken line) and 625 ng/ml (dotted line). Cell-growth is expressed as the percentage of cells (means ± range of four independent experiments) compared to uninhibited cell-growth (see Materials and Methods).



7.3.2 Effect of PTK inhibitors on P-tyr levels

Because of the differential growth-inhibiting effect of the drugs, we investigated whether the specific inhibitors affected both the level and the pattern of phosphotyrosine-containing proteins differently. Cellular proteins from cultures treated with PTK inhibitors were immunoblotted using an anti-phosphotyrosine antibody. Figure 3 shows a representative example of a P-tyr immunoblot. Herbimycin A induced a dose-dependent decrease in P-tyr levels compared to controls (lanes 2-5 versus lane 1 and 7). A 50% inhibition of P-tyr levels by herbimycin A was obtained at concentrations comparable to the IC_{50} found in the cell-growth inhibition experiments. P-tyr levels were not influenced by the other three inhibitors: RG 50875 (lane 6), RG 13022 (data not shown), and genistein (data not shown). No changes in phosphotyrosine protein patterns were noticed after incubation with any of these inhibitors.

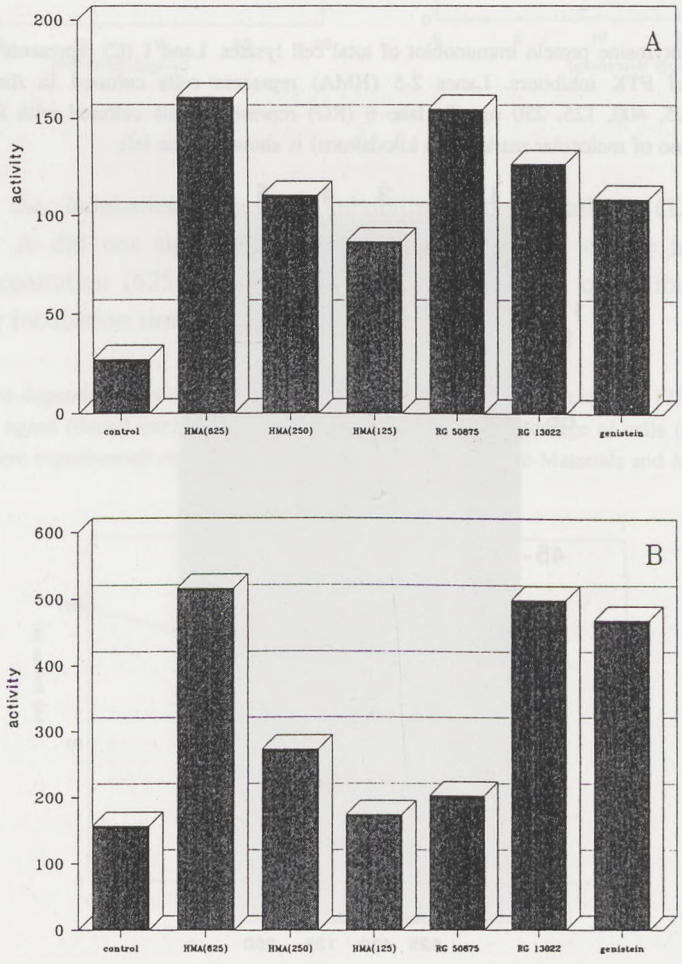
Figure 3. Phosphotyrosine protein immunoblot of total cell lysates. Lane 1 (C) represents cells cultured in the absence of PTK inhibitors. Lanes 2-5 (HMA) represent cells cultured in the presence of herbimycin A (625, 400, 125, 250 ng/ml); lane 6 (RG) represents cells cultured with RG 50875 (15 µg/ml). The position of molecular markers (in kilodaltons) is shown on the left.



7.3.3 Effect of PTK inhibitors on enzyme activities

Because of the different specificities of herbimycin A, tyrphostins, and genistein, we investigated how these inhibitors affected the overall PTK activity of cytosolic and membranous fractions of the cells. After incubation with inhibitors, cells were washed thoroughly and lyzed; subsequently, PTK activity was determined. The effect of the inhibitors on enzyme activities is shown in Figure 4. Remarkably, all inhibitors induced a rise in both cytosolic and membranous PTK activity. At least for herbimycin A, this increase was dose-dependent.

Figure 4. Influence of PTK inhibitors on cytosolic (fig. 4A) and membranous PTK activity (fig. 4B). PTK activity is expressed in pmol P-tyr/mg protein.min. Control: cells cultured in presence of vehicle only; HMA: herbimycin A (625, 250, 125 ng/ml); RG 50875 (15 µg/ml); RG 13022 (15 µg/ml); genistein (15 µg/ml).



7.4 Discussion

Protein tyrosine kinases are implicated in the regulation of growth, differentiation, and malignant transformation of cells. Inhibition of PTK activity is therefore a useful method to investigate their role in signal transduction. Furthermore, PTK activity inhibitors are potential anti-neoplastic drugs in those tumors that express high PTK activity. This is also the case where overexpression of oncogenes or growth factor receptors with PTK activity plays a role in oncogenesis.
Herbimycin A is known to reverse the altered morphologies of mammalian cells transformed with PTK oncogenes and to inhibit the *src*-kinase activity [13,43]. In human colon tumor cell lines HMA induced dose-dependent growth inhibition as well as reduction in $pp60^{c\text{-}src}$ PTK activity and $pp60^{c\text{-}src}$ steady-state protein levels [14]. It has also been shown that HMA prolongs the survival of mice inoculated with leukemic cells that demonstrate a high expression of oncogenes with PTK activity [17].
Tyrphostins are low-molecular-weight PTK inhibitors that were originally designed as substrate competitive inhibitors of EGFr kinase. They inhibit the EGF-dependent autophosphorylation of the receptor as well as the endogenous substrate tyrosine phosphorylation in intact cells [22,49]. Tyrphostins were able to retard tumor growth in a nude mice xenograft of a head and neck squamous cell carcinoma [51].
Genistein is generally used as a PTK inhibitor with rather broad specificity. In A431 cells, genistein inhibited in situ autophosphorylation of the EGFr. In vitro experiments on head and neck tumors revealed that genistein did not alter P-tyr levels [36].

Our results clearly depict the growth-inhibitory effects of herbimycin A and RG 50875 in a head and neck squamous cell carcinoma cell line. The inhibition was dose-dependent; for herbimycin A at high concentrations, it was also time-dependent. Low doses of herbimycin A induced no explicit cell-growth inhibition even after several days of incubation. RG 13022 and genistein did not induce cell-growth inhibition, even at high concentrations. Negligible cytotoxicity was found with any of the drugs used. These results show the involvement of tyrosine phosphorylation in the proliferation of this head and neck squamous cell carcinoma cell line. The most marked effects were seen using herbimycin A, indicating a possible role of *src*-family tyrosine kinases. RG 13022 and genistein, which are known to inhibit EGFr autophosphorylation, did not have an effect on tumor growth at the concentrations tested. In contrast, RG 50875, which is also supposed to inhibit EGFr kinase activity, did have an inhibitory effect. This is remarkable, as head and

neck squamous cell carcinoma cell lines are known to exhibit increased expression of EGFr, which has been associated with cell proliferation. Therefore, inhibition of EGFr autophosphorylation, which is a necessary step in its activation, is anticipated to have an effect on the EGFr kinase activity. Consequently, it is expected to have an effect on cell-growth. We have to conclude that the significance of EGFr in the proliferation of SCC-2 cells cannot be assessed by our experiments, assuming that EGFr is present in this cell line.

Increased levels of phosphotyrosine (P-tyr) have been associated with tumor growth in head and neck cancer [36,45]. P-tyr levels may be considered as a resultant of phosphorylation and dephosphorylation. Therefore, inhibition of cell proliferation in a tumor cell line model by PTK-activity inhibitors could lead to decreased P-tyr levels, at least in the presence of vanadate. Indeed, herbimycin A induced a dose-dependent decrease in P-tyr levels in SCC-2 cells. A 50% reduction of P-tyr levels was obtained at concentrations comparable with the IC_{50} in the cell-growth inhibition experiment. Thus, reduction in P-tyr levels is a reflection of decreased PTK activity during cell-growth inhibition by herbimycin A. Remarkably, we saw an overall reduction of P-tyr levels without a change in phosphoprotein pattern. This shows that all tyrosine phosphorylation of the proteins detected by this experiment is inhibited by herbimycin A. RG 50875, which also induced cell-growth inhibition in these cells, did not induce a change in P-tyr levels. Therefore, mechanisms other than reduction of total P-tyr levels may be involved in cell-growth inhibition by RG 50875.

An interesting phenomenon was observed when measuring the PTK activity after pre-incubation of the cells with the various inhibitors. After washing and lyzis of the cells, a marked increase in both cytosolic and membranous PTK activity was measured, instead of the expected decrease in kinase activity. The explanation for this phenomenon is speculative. As pointed out in the introduction, some specific PTKs normally inhibit other PTKs by phosphorylation of certain tyrosyl residues. For instance, CSK inhibits $pp60^{c\text{-}src}$ kinase activity and other *src*-family kinases by phosphorylation of a critical C-terminal tyrosyl residue. Indeed, phosphotyrosine levels in CSK-depleted model systems appear to be elevated [27]. This inactivation by phosphorylation is reversible. Reversal is accomplished by dephosphorylation of that tyrosyl residue by a specific protein tyrosine phosphatase (PTPase). In order to explain the phenomenon of increased PTK activity after treatment of cells with inhibitors, one has to realize that during this treatment, both classes of PTKs are probably inhibited. Accordingly, the *src*-like kinases, though inhibited by the presence of the drugs, are in a dephosphorylated state. Consecutive washing of the

cells, cell lyzis, and sample dilution strongly diminish the concentration of the inhibitors, thereby abolishing their direct effect on PTK activity. The result is an apparent rise in PTK activity caused by the presence of PTK forms that are still temporarily dephosphorylated at their regulatory tyrosyl residue. The finding that all PTK inhibitors induce the same effect may cast doubt on the specificity of the tyrphostins and genistein.

Though hypothetical, this rebound effect is plausible, according to present understanding of the interaction between PTKs and PTPases. To confirm this hypothesis, several experiments are conceivable. First, instead of the determination of the overall PTK activity, specific PTK activities, like $pp60^{c\text{-}src}$ activity, should be measured with an in vitro kinase assay. Second, determination of the phosphorylation state of the C-terminal tyrosyl residue by protein cleavage and subsequent immunoblotting could confirm the hypothesis posed above.

Concluding, cell-growth inhibition in a head and neck squamous cell carcinoma cell line can be achieved with PTK inhibitors. This implies that PTK inhibitors are potential anti-proliferative drugs in head and neck cancer. The fact that herbimycin A is capable of inhibiting cell-growth and reducing P-tyr levels is an indication that *src*-like tyrosine kinases play a role in the proliferation of this cell line.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

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8.1 Clinical aspects

Head and neck squamous cell carcinoma is a relatively rare disease, with respect to other tumors. It accounts for approximately 5% of all malignancies in the Netherlands. Alcohol and tobacco abuse is a major risk factor in the development of head and neck cancer [6,9]. Together with lung cancer and cancer of the esophagus, these tumors belong to the group of malignancies with a strong relationship between carcinogen exposure and malignant transformation. Ninety percent of the tumors of the upper aerodigestive tract occur in smokers and drinkers. Epidemiological and experimental data also suggest a genetic predisposition for patients developing cancers caused by environmental factors like tobacco and alcohol [5,6,10,13,18-20]. Another striking characteristic of this group is the occurrence of second primary tumors. Patients with a tumor of the head and neck, lung, or esophagus develop a second primary squamous cell carcinoma in the upper aerodigestive tract in 6-35% of the cases, either at the same time or at a later stage [6,9]. For patients with laryngeal carcinoma, the risk of developing a second primary tumor increases each year by 3% [22]. The second primaries contribute substantially to the five-year survival rate found in this patients group. For instance, patients with a glottic T1N0M0 squamous cell carcinoma are more likely to die from a second primary tumor of the lungs than from the primary tumor in the larynx [8].

Surgery and/or radiotherapy are still the primary modalities of treatment for patients with head and neck cancer. Currently, chemotherapy is only useful as adjuvant or palliative treatment, with modest results at present.
Since Billroths' first description of laryngectomy, there have been considerable improvements in the diagnosis and treatment of head and neck squamous cell carcinoma. New imaging techniques like CT scan, MRI and ultrasound-guided fine needle aspiration cytology are powerful tools in the hands of head and neck surgeons. These techniques help to localize more precisely the extent of tumors and the presence or absence of lymph node metastasis. Improvements in radiotherapy have led to less aggressive treatment modalities. This greatly reduces the destructive side effects of irradiation on the surrounding healthy tissues. In the surgical field, the use of myocutaneous flaps and especially free flaps has resulted in operations with fewer functional and aesthetic impairments. The social reintegration of patients has been facilitated by the development of various speech-restoring devices for patients who have undergone laryngectomy.
The improvements in the last few decades have undeniably been tremendously beneficial to the individual patients. Yet it remains frustrating to observe that all

these innovations have barely been able to improve the overall survival rate of patients with head and neck cancer. Analysis of clinical data has led to the detection of several prognostic parameters like tumor size and localization, lymph node metastasis, and histological features. Treatment modalities of patients are consequently chosen according to these clinicopathological parameters. However, individual prognosis is hard to make with these parameters [1]. Patients with the same prognostic parameters can have a totally different disease behavior.

Therefore, basic research for prognostic molecular parameters is mandatory. New molecular parameters could also be used as targets for detection of micrometastases or for treatment with new cytotoxic drugs [3]. Another important aim of basic research is the development of molecular markers for the occurrence of second primary tumors.

8.2 Biochemical aspects

Phosphorylation and dephosphorylation of proteins are considered to be the main mechanisms controlling signal transduction of the cells [7,23]. Derangement of the balance between these processes could lead to malignant transformation. The study of protein tyrosine kinases (PTK) has implicated the phosphorylation of tyrosyl residues in proteins as an essential element in the control of both normal and neoplastic cell growth. However, this furnishes an incomplete picture of the reversible and dynamic process of protein tyrosine phosphorylation. The characterization of the role of protein tyrosine phosphatases (PTPase) provides a complementary perspective from which to achieve an overall understanding of the control of cellular function by tyrosine phosphorylation. The levels of phosphotyrosine found in the proteins of the normal and neoplastic cells are tightly regulated by the opposing actions of PTKs and PTPases. Increases in phosphotyrosine are associated with the responses of cells to numerous external stimuli [2,21,24]. Under normal conditions, such phosphorylations are transient and are reversed by the actions of PTPases. The observation that unregulated tyrosine phosphorylation can result in malignant transformation also raises the possibility that aberrant tyrosine dephosphorylation may also confer uncontrolled cell growth. Unravelling of the precise interactions between protein tyrosine kinases and protein tyrosine phosphatases, which are responsible for phosphorylation and dephosphorylation, would be a major step forward in our understanding of neoplastic transformation.

Protein tyrosine kinases can be classified into two groups: the receptor kinases and the non-receptor kinases. The function of the receptor kinases is unambiguous; they are responsible for transducing signals provided by ligand binding to their external domain into cytoplasmatic second messengers to ultimately reach the cell nucleus [4,11]. The epidermal growth factor receptor (EGFr) is a well-known example of this class of protein tyrosine kinase in head and neck cancer. Binding of EGF or transforming growth factor α (TGFα) to EGFr results in a dimerization of the receptor and an activation of the intracellular protein tyrosine kinase activity. Autophosphorylation or heterologous phosphorylation of the receptor is probably needed to exert the protein tyrosine kinase activity [11,15].
The functions of the non-receptor kinases, with most members of this group belonging to the *src*-family of oncogenes, are less well understood. The *src*-product $pp60^{c\text{-}src}$ has been associated with the *ras*-oncogene product and with activated growth factor receptors [14,16,17,25]. Therefore, it is generally accepted that non-receptor kinases are elements of the signal transduction chain from growth factor receptor to cell nucleus [12,26]. $Pp60^{c\text{-}src}$ is regulated by autophosphorylation as well as by phosphorylation of a specific tyrosyl residue. Phosphorylation of $pp60^{c\text{-}src}$ by a special cytosolic tyrosine kinase results in inactivation of the kinase activity and thus offers a potential activation mechanism by PTPase. In human lymphoid cells, such a PTPase has already been identified and appears to be a membrane-bound receptor-like protein.

As both receptor and non-receptor PTKs have specific phosphorylation sites which can activate or de-activate the kinase activity, it is likely that protein tyrosine phosphatases are more than enzymes which counteract the phosphorylation of proteins. Therefore the phosphorylation state of proteins is probably the result of a subtle balance between PTKs and activating or inhibiting PTPases. Nevertheless, presumably the main action of the PTPases is still to counteract the effect of PTKs. At present, only fragmentary information is available on the exact role of PTPases in signal transduction.

As mentioned before, the search for molecular parameters is essential to improvement of the clinical outcome and fundamental understanding of head and neck cancer. Much of the basic research in head and neck cancer has been focused on oncogenes and tumor suppressor genes. Many oncogenes code for products with PTK activity, and many growth factor receptors also exhibit protein tyrosine kinase activity. Growth factor receptors - especially epidermal growth factor receptor (EGFr) - have been extensively investigated in head and neck cancer. Therefore,

understanding of the role of phosphorylation in head and neck cancer may lead us towards useful molecular parameters.

8.3 General review of the thesis

Chapter 2 presents an overview of published research on oncogenes, tumor suppressor genes, and growth factor receptors in head and neck cancer. The overview emphasizes the role these biochemical parameters play in signal transduction of the malignant cell.

Chapter 3 presents the results of a pilot study on PTK activity in laryngeal cancer. We found a statistically significant increase in cytosolic PTK activity in tumor tissue compared to non-tumor tissue of cancer patients as well as of control patients. Remarkably, we also found a significant increase in cytosolic PTK activity in, histologically proven, non-tumor tissue of cancer patients compared to control tissue. Therefore, we may conclude that non-tumor tissue shows premalignant enzymological changes that are not reflected in histological changes. We do not believe that these changes are a direct consequence of tobacco and/or alcohol consumption alone, since we have shown that the PTK activity in control mucosa is unaffected by smoking and/or drinking habits.

In Chapter 4, we further explored the role of cytosolic PTK activity in tumor and non-tumor tissues. We also determined the cytosolic PTPase activity in these tissues. PTK and PTPase activities were elevated in tumor tissues compared to, histologically proven, non-tumor mucosa of the cancer patients as well as controls. Additionally, PTK activity in non-tumor tissue of cancer patients was significantly higher than in normal tissue of the control group. The same trend was observed for the PTPase activity. Furthermore, we determined the patterns of tyrosine phosphorylated protein (P-tyr) in tissues by Western blotting. Meaningful results were only obtained when PTPase activity was completely blocked by the addition of the PTPase inhibitor orthovanadate. In the absence of vanadate, no phosphotyrosyl-containing proteins could be detected, neither in tumor nor in non-tumor or control tissues. Obviously, in vitro PTK activity is overruled by PTPase activity. P-tyr levels in tumors were higher than in non-tumor and control tissues. P-tyr levels in non-tumor tissues were also increased in non-tumor tissue compared to controls. No significant differences in protein patterns were seen among the tumor, non-tumor, and control tissues.

In Chapter 5 we investigated whether the PTPase activity could be detected with an enzymehistochemical technique. We found that PTPase activity was easily detected with light microscopy. We found increased PTPase activities in tumor cells as compared to control mucosa. To a lesser extent, PTPase activities were elevated in non-tumor tissue compared to control tissue. We also found high activities in the surrounding connective tissue. This activity was mainly localized in the collagen fibers, not in the fibroblasts, and must be considered as an aspecific reaction of collagens with lead salts. Orthovanadate, a PTPase inhibitor, strongly inhibited the enzyme activity in all tissues. No staining was seen in the absence of phosphotyrosine or in the presence of phosphoserine or phosphothreonine. Therefore this method must be considered as specific for PTPase activity.

In Chapter 6 we correlated the cytosolic and membrane-bound PTK and PTPase activity of tumor and non-tumor tissue with several clinical and histological parameters that are known to influence the clinical outcome. Furthermore, we evaluated the enzyme activities as independent predictors of clinical behavior, recurrence, and occurrence of second primary tumors.

We confirmed our earlier observations that cytosolic as well as membranous PTK and PTPase activities in tumor tissues are increased compared to non-tumor tissues and controls. Moreover, we also confirmed the findings of increased enzyme activities in, histologically proven, non-tumor mucosa of cancer patients compared to control tissues. This finding is highly interesting, as it indicates that these biochemical changes are obviously not (yet) translated into morphological changes. An explanation could be an enzymological premalignant state of the non-tumor mucosa. Therefore, it could have a predictive value for the occurrence of second primary tumors. A paracrine effect is less likely, due to the relatively large distance between tumor and non-tumor samples. It is not a direct effect of carcinogenic factors; we have shown in Chapter 3 that alcohol and tobacco have no influence on enzyme activities of the controls. Unfortunately, we did not find any significant differences in enzyme activities in non-tumor tissue in patients who developed a second primary compared to those who did not. However, the incidence of second primary tumors in our series is 5%. Moreover, the patient group is small (n=107) and the follow-up of the whole patient group is still relatively short (median 25.4 months). For these reasons, definitive conclusions about the predictive value of increased enzyme activities in non-tumor mucosa can only be drawn after several years.

Another explanation could be that the altered enzyme activities are indications for increased mutagen sensitivity of the head and neck mucosa. In peripheral blood lymphocytes of patients with head and neck cancer, an increased sensitivity to bleomycin-induced chromosomal damage has been reported [10,20]. This mutagen sensitivity is clearly a genetic trait, as it occurs in cells not related to the tumor or exposed to carcinogens. Mutagen sensitivity could play a significant role in oncogenesis in tissues that are in contact with environmental factors. It may explain why some patients with a history of alcohol and tobacco abuse develop a cancer of the upper aerodigestive tract while other patients do not. It is tempting to speculate that increased PTK and PTPase activity in non-tumor tissue are a reflection of the increased mutagen sensitivity of cancer patients' mucosa for tobacco and alcohol. Whatever the explanation of the altered enzyme activities in non-tumor mucosa, it is an exciting finding. This observation certainly warrants further research to evaluate its significance as a prognostic parameter.

In order to assess the significance of enzyme activity determination as a predictor for clinical behavior, we analyzed the survival and disease-free interval of the patients. We could not find a relation with any of the enzyme activities measured. Admittedly, the follow-up is still short. But as the survival and disease-free curves of patients with high and low enzyme activities are nearly synchronous, we do not expect to see significant differences in a longer follow-up. Therefore, we may assume that the measurement of the activities of these enzymes does not make a meaningful contribution to the prediction of clinical outcome.

Notably, we did find a relation between membranous PTK activity and localization, lymph node metastasis, sex, and previous radiotherapy. Especially interesting was the increased PTK activity in lymph node positive patients. High expression of EGFr, which exhibit membranous PTK activity, has frequently been reported in head and neck cancer. In other tumors, membrane-bound *src*-kinase activity was frequently elevated as compared to normal tissues. Increased membranous PTK activity may therefore be a reflection of increased activity of EGFr kinase or *src*-kinase, amongst others. This could mean that EGFr kinase or *src*-kinase activity is higher in metastatic head and neck cancer. Of course, it would be premature to conclude that those proteins are responsible for metastasis, but it is tempting to do so.

We were surprised to find that membranous PTK activity in previously treated patients was decreased, as compared to untreated patients. All these patients had

previously received radiotherapy before the enzyme activities in the recurrent tumor were analyzed. The outcome may be a selecting effect of radiotherapy, in the sense that cells with low membranous PTK activity are more radioresistant than other cells. Therefore, surviving cell clones would demonstrate lower membranous PTK activity.
Interestingly, we found significant differences in non-tumorous tissues of female and male patients. All enzyme activities measured, except for the membranous PTPase activity, were higher in females than in males. This was surprising, as these sex differences were not found in the control patients. It is, of course, too early to known whether this means that the female patients in this series will develop more second primaries than the men. Unfortunately, we could not find clinical reports documenting a higher incidence of second primaries in female patients.

Chapter 7 further examines the role of protein tyrosine kinases in head and neck cancer by means of a tumor cell line model. We questioned whether the inhibition of PTK activity could influence the growth of a head and neck squamous cell carcinoma cell line.
To investigate this, we used cultured cells derived from human head and neck cancer and inhibited them with several known PTK inhibitors. The outcome revealed how PTK inhibition altered the cell growth. We also evaluated how PTK activities and levels of tyrosyl phosphorylation in the cell lines were influenced by the PTK inhibitors.
Two of the four PTK inhibitors, herbimycin A and tyrphostin RG 50875, showed dose-dependent cell growth inhibition. Two other PTK inhibitors, tyrphostin RG 13022 and genistein, had no effect on cell growth. Herbimycin A, that specifically inhibits *src*-like tyrosine kinases, also had a dose-dependent influence on the phosphotyrosine levels in the cells. The growth-inhibiting tyrphostin RG 50875 as well as the non-inhibiting tyrphostin RG 13022 and genistein had no effect on the phosphotyrosine levels in the cells. No changes in phosphotyrosine protein patterns were seen in any of the inhibitors.

An interesting phenomenon was observed when measuring the PTK activity after pre-incubation of the cells with the various inhibitors. After washing and lysis of the cells, a marked increase in both cytosolic and membranous PTK activity was measured instead of the expected decrease in kinase activity. The explanation for this phenomenon is speculative. As pointed out in Chapter 2, some specific PTKs normally inhibit other PTKs by phosphorylation of certain tyrosyl residues. This inactivation by phosphorylation is reversible through the dephosphorylation of that

tyrosyl residue by a specific protein tyrosine phosphatase (PTPase). In order to explain the phenomenon of increased PTK activities after treatment of cells with inhibitors, one has to realize that during this treatment both classes of PTKs are probably inhibited. By consecutive washing of the cells, cell lysis, and sample dilution, the concentration of the inhibitors is strongly diminished. Thereby the direct effect on PTK activity is abolished. The result is an apparent increase of PTK activity due to the presence of PTK forms which are still temporarily dephosphorylated at their regulatory tyrosyl residue.
This rebound effect is hypothetical. Yet it may be plausible, according to the present understanding of the interaction between PTKs and PTPases. To confirm this hypothesis, several experiments are proposed.

Concluding, cell growth inhibition in a head and neck squamous cell carcinoma cell line can be achieved with PTK inhibitors. This implies that PTK inhibitors are potential anti-proliferative drugs in head and neck cancer. The fact that herbimycin A is capable of inhibiting cell growth and reducing P-tyr levels is an indication that *src*-like tyrosine kinases play a role in the proliferation of this cell line.

8.4 Conclusions

1. In head and neck squamous cell carcinoma, cytosolic and membrane-bound PTK and PTPase activities are increased compared to non-tumor tissue of cancer patients and controls.

2. Non-tumor mucosa of patients suffering from head and neck cancer show an increase in PTK and PTPase activities compared to control mucosa of healthy persons. Non-tumor mucosa of head and neck cancer patients can be considered as enzymologically premalignant.

3. Membrane-bound PTK activity of tumor tissue is related to sex, localization, previous treatment, and lymph node metastasis. Enzyme activities in tumor tissue are not useful as prognostic parameters. It is too early to say whether enzyme activities in non-tumor tissue can be useful as a predictor for the occurrence of second primary tumors. Alcohol and tobacco abuse does not have an influence on enzyme activities of either tumor or non-tumor tissues.

4. The level of phosphorylated tyrosyl residues on proteins is increased in tumor tissue compared to non-tumor and control tissue. In non-tumor tissue, phosphotyrosine levels are also increased compared to controls.

5. Enzymehistological determination of PTPase activity in head and neck cancer is simple and reproducible. Increased PTPase activities were found in tumor cells and to a lesser extent in non-tumor cells compared to control tissue. Increased PTPase activity was also found on collagens in the surrounding connective tissue.

6. The PTK inhibitors herbimycin A and tyrphostin RG 50875 show dose-dependent cell growth inhibition in cultured head and neck squamous cell carcinoma cells. PTK inhibitors can be considered as potential anti-proliferative drugs in head and neck cancer.

7. The fact that herbimycin A induces dose-dependent growth inhibition as well as a reduction in phosphotyrosine levels is an indication that *src*-like tyrosine kinases play an important role in cell proliferation of a head and neck squamous cell carcinoma cell line.

8. An increase in PTK activity was noted after previous incubation with PTK inhibitors. This rebound effect is an indication that these drugs (also) inhibit specific phosphorylation of sites on PTKs which are needed for kinase activation.

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SUMMARY

Summary

Head and neck squamous cell carcinoma accounts for approximately 5% of all malignancies in The Netherlands. Alcohol and tobacco abuse is known to be a major risk factor in the development of head and neck cancer. Epidemiological and experimental data also suggest a genetic predisposition. Another striking characteristic of this group is the occurrence of second primary tumors. At present, surgery and/or radiotherapy are the primary modalities of treatment for patients with head and neck cancer. But despite diagnostic and therapeutic innovations in the last decades, the overall survival rate of patients with head and neck cancer has barely improved. While clinical and histological parameters are able to predict the prognosis of specific subpopulations of patients, individual outcome is hard to foresee. Therefore, the search for new molecular parameters is essential in improving the clinical outcome as well as in gaining a fundamental understanding of head and neck cancer.

One of the important issues in cancer research, which is reviewed in Chapter 2, is how a malignant cell transmits the 'oncogenic' signals through the cell. Phosphorylation and dephosphorylation of tyrosine residues on proteins are considered to be the main mechanisms controlling signal transduction of the cells. Derangement of the balance between these processes could lead to malignant transformation. Phosphorylation is catalyzed by protein tyrosine kinases (PTKs) and dephosphorylation by protein tyrosine phosphatases (PTPases). The effect of these enzymes is generally antagonistic. Yet a complex interaction exists between these enzymes, leading to mutual inhibition or activation of their activities. Protein tyrosine kinases can be classified into two groups: the receptor kinases and the non-receptor kinases. The function of the receptor kinases is unambiguous; they are responsible for transducing signals, which are provided by ligand binding to their external domain, into cytoplasmatic second messengers that ultimately reach the cell nucleus. Non-receptor kinases are elements of the signal transduction chain from growth factor receptor to cell nucleus. Many oncogene products possess PTK activity. At present, the available information on the exact role of PTPases in signal transduction is limited.

In this thesis, the presence and activities of PTKs and PTPases have been investigated in head and neck cancer tissue, non-tumor tissue of cancer patients, and controls. In Chapter 3, 4, and 6 is demonstrated that both the cytosolic and the membranous PTK and PTPase activities of tumor cells are increased as compared to non-tumor tissue of cancer patients and controls. Furthermore, non-tumor mucosa of

patients suffering from head and neck cancer showed an increase in PTK and PTPase activities compared to control mucosa of healthy persons. Non-tumor mucosa of head and neck cancer patients can therefore be considered as enzymologically premalignant.

In Chapter 6, correlations have been made between enzyme activities on the one hand and clinico-pathological parameters and clinical outcome on the other hand. The membrane-bound PTK activity of tumor tissue was found to be related to sex, localization, previous treatment, and lymph node metastasis. However, the determination of enzyme activity in tumor tissue was not useful as a independent prognostic parameter for clinical outcome. It is too early to say whether enzyme activities in non-tumor tissue can be used as a predictor for the occurrence of second primary tumors. There was no clear correlation between alcohol and tobacco abuse on the one hand with enzyme activities of either tumor or non-tumor tissues on the other.

In Chapter 4, the pattern of tyrosine phosphorylated proteins in the cytosolic fractions of tumorous, non-tumorous, and control samples was investigated as a direct measurement of the balance between PTK and PTPase activity. Phosphorylation of endogenous proteins, with subsequent electrophoresis and immunoblotting, revealed several phosphotyrosyl-containing proteins with apparent molecular weights ranging from 33 to 120 kD. The level of phosphorylated tyrosyl residues on proteins was found to be increased in tumor tissue compared to non-tumor and control tissue. In non-tumor tissue, phosphotyrosine levels were also increased compared to controls. No significant differences in protein patterns were seen among the tumor, non-tumor, and control tissues. However, meaningful results were only obtained when PTPase activity was completely blocked by the addition of the PTPase inhibitor orthovanadate. In the absence of orthovanadate, no phosphotyrosyl-containing proteins could be detected, either in tumor or in non-tumor, nor in controls. Obviously, in vitro PTK activity is overruled by PTPase activity.

In Chapter 5, it is examined whether the PTPase activity could be detected with an enzymehistochemical technique. PTPase activity was found to be easily detected with light microscopy. Increased PTPase activities were found in tumor cells as compared to control mucosa. To a lesser extent, PTPase activities were also elevated in non-tumor tissue compared to control tissue. Orthovanadate strongly inhibited the enzyme activity in all tissues. No staining was seen in the absence of

phosphotyrosine or in the presence of phosphoserine or phosphothreonine. Therefore, this method must be considered as specific to PTPase activity.

Chapter 7 addressed the question whether or not specific PTK inhibitors can influence cell growth, enzyme activities, and phosphotyrosine levels in a head and neck squamous cell carcinoma cell line. The PTK inhibitors herbimycin A and tyrphostin RG 50875 showed dose-dependent cell growth inhibition. PTK inhibition can therefore be considered as a potential mechanism for new anti-proliferative drugs in head and neck cancer.
The fact that herbimycin A, which is considered to have some specificity for *src*-kinases, induces dose-dependent growth inhibition as well as a reduction in phosphotyrosine levels is revealing. It is an indication that *src*-like tyrosine kinases play an important role in cell proliferation of a head and neck squamous cell carcinoma cell line.

Finally, in Chapter 8, the results of these investigations are summarized and discussed.

RESUME

Résumé
Les carcinomes épidermoïdes cervico-faciales représentent environ 5% de tous les cancers aux Pays-Bas. Le tabagisme et l'éthylisme sont des facteurs de risque importants dans les cancers cervico-faciales. Des données épidémiologiques et expérimentales suggèrent également une prédisposition génétique. Une autre caractéristique marquante est la présence de multiples tumeurs des voies aéro-digestives supérieures. Actuellement, la chirurgie et/ou la radiothérapie constituent le traitement de choix. Malgré les innovations diagnostiques et thérapeutiques de ces dernières décades, la mortalité par cancers cervico-faciales n'a guère diminué.
Tandis que certains paramètres cliniques et histologiques sont capables d'estimer le pronostic de certains groupes de malades, le pronostic individuel est difficile à établir. Ainsi, il est essentiel de chercher de nouveaux paramètres moléculaires pour améliorer les résultats cliniques et pour obtenir une meilleure compréhension fondamentale du cancer cervico-facial.

Un des sujets majeur dans la recherche cancérologique, lequel est décrit dans le deuxième Chapitre, est comment une cellule maligne transmet son signal oncogène à travers la cellule. La phosphorylation et déphosphorylation de la tyrosine protéique est considerée comme étant un des mécanismes les plus importants dans la transmission des signaux cellulaires. Un deséquilibre entre ces processus enzymologiques pourrait mener à une transformation maligne. La phosphorylation est catalysée par les protéine tyrosine kinases (PTKs) et la déphosphorylation par les protéine tyrosine phosphatases (PTPases). L'effet de ces enzymes est généralement antagonique mais il existe une interaction complexe entre ces enzymes avec une inhibition ou activation mutuelle de leurs activités. Les protéine tyrosine kinases peuvent être réparties en deux catégories: les kinases récepteurs et les kinases non-récepteurs. La fonction des kinases récepteurs est évidente: elles sont responsables de transmettre des signaux, générés par l'attachement de ligands à leurs domaines extracellulaires, vers des messagers intracellulaires secondaires pour atteindre finalement le noyau. Les kinases non-récepteurs sont des éléments de la chaine de transmission de signaux, du recepteurs de facteur de croissance au noyau. Nombre de produits d'oncogènes possèdent une activité de PTK. Actuellement, l'information disponible sur le role exact des PTPases dans la transmission de signaux est limitée.

Dans cette thèse la présence et l'activité des PTKs et PTPases ont été étudiées dans les tissus cancéreux et les muqueuses non-cancéreuses de malades atteints de carcinomes épidermoïdes cervico-faciales ainsi que dans les muqueuses de sujets contrôles. Dans les Chapitres 3, 4 et 6 est démontré que les activités des PTKs et

des PTPases cytosolaires et membraneuses des tissus cancéreux sont élevées comparé aux tissus non-cancéreux et aux contrôles. Les muqueuses non-cancéreuses de malades cancéreux montraient une augmentation des activités des PTKs et des PTPases comparé aux tissus de contrôles. Les muqueuses non-cancéreuses peuvent ainsi être considerées comme étant precancéreux du point de vue enzymologique.

Dans le Chapitre 6 nous décrivons les corrélations entre les activités enzymologiques d'une part et certains paramètres cliniques et histologiques et résultats cliniques d'autre part. L'activité membraneuse des PTKs dans les tissus cancéreux était corrélé au sexe, à la localisation, au traitement antérieur et à la présence de métastases ganglionnaires. Cependant, la détermination des activités enzymologiques n'est pas valable comme nouveau paramètre de pronostic. A présent, il est prématuré de conclure que la détermination des activités enzymologiques peut avoir une valeur pronostique dans le développement des multiples tumeurs cervico-faciales. Il n'éxiste pas de corrélations étendues entre le tabagisme et l'éthylisme d'une part et les activités des enzymes dans les tissus cancéreux et non-cancéreux d'autre part.

Dans le Chapitre 4 le spectre des phosphotyrosines protéiques a été étudié dans les tissus cancéreux et non-cancéreux ainsi que dans les contrôles. Ce pour avoir une impression sur le résultat de l'equilibre entre les PTKs et PTPases. La phosphorylation de protéines endogènes, suivi d'électrophorèse et d'immunoblotting, a démontré plusieurs protéines avec un poids moléculaire entre 33 et 120 kD. Le quantité de phosphotyrosines protéiques était plus élevée dans les tissus cancéreux que dans les tissus non-cancéreux ou dans les contrôles. Il n'existait pas de différences significatives dans les spectres protéiques des différents tissus. Des résultats valables n'étaient cependant seulement obtenus si l'activité des PTPases était totalement bloquée par l'orthovanadate, un inhibiteur de PTPases. Dans l'absence d'orthovanadate il n'était pas possible de détecter de protéines phosphorylées. Apparemment, l'activité des PTKs in vitro est dominée par l'activité des PTPases.

Dans le Chapitre 5 est décrit dans quelle mesure l'activité des PTPases peut être déterminée avec une méthode enzymo-histochimique. Manifestement, l'activité des PTPases est facilement détectable avec la phosphotyrosine comme substrat. Les cellules cancéreuses montraient une augmentation d'activité de PTPase comparé aux muqueuses normales. L'activité de PTPase dans les muqueuses hyperplasiques était légèrement élevée par rapport aux muqueuses normales. L'addition d'orthovanadate

résultait en une inhibition majeure des réactions dans les différents tissus. Aucune réaction n'était détectable dans l'absence de phosphotyrosine ou dans la présence de phosphoserine ou phosphothréonine comme substrat. Cette méthode doit ainsi être considerée comme étant spécifique pour la détection de l'activité de PTPases.

Dans le Chapitre 7 la question est posée si des inhibiteurs spécifiques de PTK sont capable d'influencer la croissance, les activités enzymologiques et la quantité de phosphotyrosine dans des cultures cellulaires de cancer épidermoïde cervico-faciale. Les inhibiteurs de PTK herbimycine A et tyrphostin RG 50875 induisaient une diminution de croissance de ces cultures cellulaires qui était dependante de la concentration utilisée. L'inhibition de l'activité de PTK peut ainsi être considerée comme un point de départ potentiel pour le développement de nouvelles substances cytostatiques. Le fait que herbimycine A, qui est rélativement spécifique pour les kinases de la famille de *src*, donne une diminution de la croissance ainsi qu'une baisse de la quantité de phosphotyrosine, est une indication que les kinases de la famille de *src* jouent un rôle important dans la prolifération des ces cultures cellulaires épidermoïdes.

Finalement, les résultats de ces expériences sont résumés et commentés dans le Chapitre 8.

SAMENVATTING

Samenvatting

Hoofd-hals plaveiselcel carcinomen vertegenwoordigen ongeveer 5% van alle maligniteiten in Nederland. Alcohol en tabak misbruik zijn belangrijke risicofactoren in het ontstaan van hoofd-hals tumoren. Epidemiologische en experimentele gegevens wijzen ook op een bepaalde genetische predispositie. Een ander opmerkelijke eigenschap van deze tumoren is het voorkomen van tweede primaire tumoren. Op dit moment zijn chirurgie en/of radiotherapie de voornaamste therapeutische mogelijkheden voor patiënten met hoofd-hals tumoren. Maar ondanks diagnostische en therapeutische vernieuwingen in de afgelopen tientallen jaren, is de overlevingskans van patiënten met hoofd-hals tumoren nauwelijks verbeterd. Daar waar klinische en pathologische parameters in staat zijn om de prognose van specifieke patiënten groepen te voorspellen, is de individuele prognose moeilijk in te schatten. Daarom is het essentieel om nieuwe moleculaire parameters te zoeken teneinde de klinische resultaten te verbeteren en om een beter fundamenteel inzicht te krijgen in hoofd-hals kanker.

Een van de belangrijke onderwerpen in kanker onderzoek, welke in Hoofdstuk 2 besproken wordt, is hoe een maligne cel zijn oncogeen signaal in de cel doorgeeft. Fosforylering en defosforylering van tyrosine residuen in eiwitten wordt beschouwd als één van de belangrijkste mechanismen in de signaal transductie van de cel. Ontregeling van het evenwicht tussen deze processen zou kunnen leiden tot maligne transformatie. Fosforylering wordt gekatalyseerd door proteïne tyrosine kinases (PTKs) en defosforylering door proteïne tyrosine fosfatases (PTPases). Het effect van deze enzymen is in het algemeen tegengesteld maar er bestaat een complexe interactie tussen deze enzymen welke tot wederzijdse inhibitie of activatie van hun activiteiten leidt. Proteïne tyrosine kinases kunnen onderverdeeld worden in twee groepen: de receptor kinases en de non-receptor kinases. De functie van de receptor kinases is evident: zij zijn verantwoordelijk voor het doorgeven van signalen, die gegenereerd worden door het binden van een ligand aan hun extracellulaire domein, naar intracellulaire boodschapper moleculen om uiteindelijk de celkern te bereiken. Non-receptor kinases zijn elementen van de signaal transductie keten van groei factor receptor naar celkern. Veel oncogen produkten bezitten PTK activiteit. Op dit moment is de beschikbare informatie over de exacte rol van PTPases in de signaal transductie nog beperkt.

In dit proefschrift is de aanwezigheid en activiteit van PTKs en PTPases onderzocht in hoofd-hals tumoren, non-tumor weefsel van kanker patiënten en controle personen. In Hoofdstuk 3, 4 en 6 wordt aangetoond dat zowel de cytosolaire als de

membraneuze PTK en PTPase activiteiten van tumor weefsel verhoogd waren ten opzichte van non-tumor weefsel van kanker patiënten en van controles. Tevens liet non-tumor mucosa van kanker patiënten verhoogde PTK en PTPase activiteiten zien in vergelijking tot controle weefsel van gezonde proefpersonen. Non-tumor mucosa van patiënten met een hoofd-hals tumor kan daardoor beschouwd worden als enzymologisch premaligne.

In Hoofdstuk 6 zijn er correlaties gezocht tussen enerzijds enzym activiteiten en anderzijds klinische en pathologische parameters en klinisch beloop. De membraneuze PTK activiteit van tumor weefsel bleek gerelateerd te zijn met geslacht, localisatie, eerdere behandeling en lymfeklier metastasen. Echter, de bepaling van enzym activiteit in tumor weefsel was niet bruikbaar als nieuwe onafhankelijke prognostische parameter voor het klinisch beloop. Op dit moment is het nog te vroeg om te stellen of enzym activiteiten in non-tumor weefsel een voorspellende waarde hebben voor het ontstaan van tweede primaire tumoren. Er werden geen duidelijke correlaties gevonden tussen alcohol en tabak gebruik enerzijds en enzym activiteiten in tumor en non-tumor weefsel anderzijds.

In Hoofdstuk 4 werd het patroon onderzocht van aan tyrosine gefosforyleerde eiwitten in de cytosolaire fracties van tumor, non-tumor en controle weefsel. Dit werd gedaan om een direct inzicht te hebben in het resultaat van het evenwicht tussen PTK en PTPase activiteit. Fosforylering van endogene eiwitten, met aansluitend elektroforese en immunoblotting, liet verschillende fosfotyrosyl bevattende eiwitten zien met een moleculair gewicht variërend tussen 33 en 120 kD. Het gehalte van gefosforyleerde tyrosyl residuen op eiwitten was hoger in tumor weefsel dan in non-tumor of in controle weefsel. In non-tumor weefsel waren de fosfotyrosine gehaltes verhoogd in vergelijking tot de controles. Er werden geen significante verschillen gevonden in eiwit patroon tussen de verschillende weefsels. Bruikbare resultaten werden echter alleen verkregen als de PTPase activiteit volledig geblokkeerd werd door de PTPase remmer orthovanadaat. In de afwezigheid van orthovanadaat werden er geen fosfotyrosine bevattende eiwitten gedetecteerd in de verschillende weefsels. Kennelijk wordt de PTK activiteit in vitro overheerst door de PTPase activiteit.

In Hoofdstuk 5 wordt beschreven in hoeverre PTPase activiteit bepaald kan worden door middel van een enzym-histochemische methode. PTPase activiteit bleek lichtmicroscopisch makkelijk waarneembaar te zijn met fosfotyrosine als substraat. Tumor cellen bleken toegenomen PTPase activiteiten te vertonen ten opzichte van

gezonde controle mucosa. De PTPase activiteiten in hyperplastische mucosa bleken eveneens in lichte mate verhoogd te zijn in vergelijking tot (normale) controle mucosa. Orthovanadaat gaf een sterke remming van de reactie in alle weefsels. Er werd geen kleuring gevonden in afwezigheid van fosfotyrosine of in aanwezigheid van fosfoserine of fosfothreonine. Deze methode moet daarom beschouwd worden als specifiek voor PTPase activiteit.

In Hoofdstuk 7 wordt de vraag gesteld of specifieke PTK remmers in staat zijn om de celgroei, de enzym activiteiten en de fosfotyrosine gehaltes te beïnvloeden in een hoofd-hals plaveiselcel carcinoom cellijn. De PTK remmers herbimycine A en tyrphostin RG 50875 lieten een dosis afhankelijke celgroei remming zien. Remming van PTK activiteit kan dus beschouwd worden als een potentieel aanknopingspunt voor nieuwe cytostatische middelen. Het feit dat herbimycine, welke deels specifiek is voor *src*-kinases, een dosis afhankelijke groei remming geeft en ook een afname in fosfotyrosine gehaltes is een aanwijzing dat *src*-achtige tyrosine kinases een belangrijke rol spelen in de cel proliferatie van deze hoofd-hals plaveiselcel carcinoom cellijn.

Tot slot worden in Hoofdstuk 8 de resultaten van al deze onderzoeken samengevat en van commentaar voorzien.

DANKWOORD

Tableau de la troupe

Een promotie is als een toneelstuk waarin de promovendus niet alleen het script heeft geschreven maar ook de hoofdrol speelt. Net als bij elke toneelvoorstelling zijn ook hier vele mensen geweest die op creatieve wijze hebben bijgedragen aan het resultaat.

De artistiek leider van het gezelschap, prof. dr. G.E.J. Staal: beste Gerard, jouw onbegrensd enthousiasme voor elke produktie die jouw gezelschap weer op de planken zet, draagt in hoge mate bij aan het applaus na elke voorstelling. Als je op dezelfde manier de Faculteit kan stimuleren als jouw vakgroep ben ik overtuigd dat haar toekomst bloeiend zal zijn.

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Toneelgezelschappen zijn nergens zonder juiste technische ondersteuning.

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Tot slot, het doel van elk toneelstuk is de boodschap die men wil overbrengen. Deze boodschap is in feite slechts bedoeld voor één persoon welke tevens de reden van bestaan voor deze schrijver is. Echter door haar te benoemen en te bedanken, wordt de waarde die ze voor hem vertegenwoordigt gebanaliseerd. Daarom aan het eind van dit script geen banale woorden maar een open einde.....

CURRICULUM VITAE

The author was born on March 16th 1961 in Eindhoven, The Netherlands. After obtaining his Athenaeum diploma in "Het Nieuwe Lyceum" in Hilversum in 1978, he started his medical study at the University of Groningen. During his study he worked as a "hartejongen" at the cardiosurgical intensive care of the University Hospital Groningen. Before graduating, he did some research on the clinical application of the electroglottographe at the "Fondation A. de Rotschild" in Paris (head: Prof. P. Elbaz) and on sound localization at the "Hôpital Avicenne" in Bobigny, France (head: Prof. B. Frachet). After working at the department of General Surgery of the "St. Lukas Ziekenhuis" in Amsterdam (head: Dr. J.N. Keeman) for one year, he obtained a clinical fellowship ("médecin resident étranger") from the "College de Médecine" in Paris. This fellowship was fulfilled at the department of Otorhinolaryngology of the "Hôpital Avicenne" in Bobigny, Université Paris XIII, France (head: Prof. B. Frachet). Besides clinical activities, the author did some research on electrically evoked brainstem response audiometry in cochlear implant patients. He also participated during his fellowship in a medical research expedition on the Mont Blanc to investigate the influence of acute mountain sickness on audiological and vestibular functions. In April 1989 he started his residency at the department of Otorhinolaryngology of the University Hospital Utrecht (head: Prof. dr. E.H. Huizing). Six months of his training were realized at the "Lucas Ziekenhuis" in Apeldoorn (head: Dr. J. Antvelink). The author became an otorhinolaryngologist on April 1th 1994. For two years, he will be working as a clinical fellow in head and neck oncology for the Dutch Cancer Institute ("Koningin Wilhelmina Fonds").