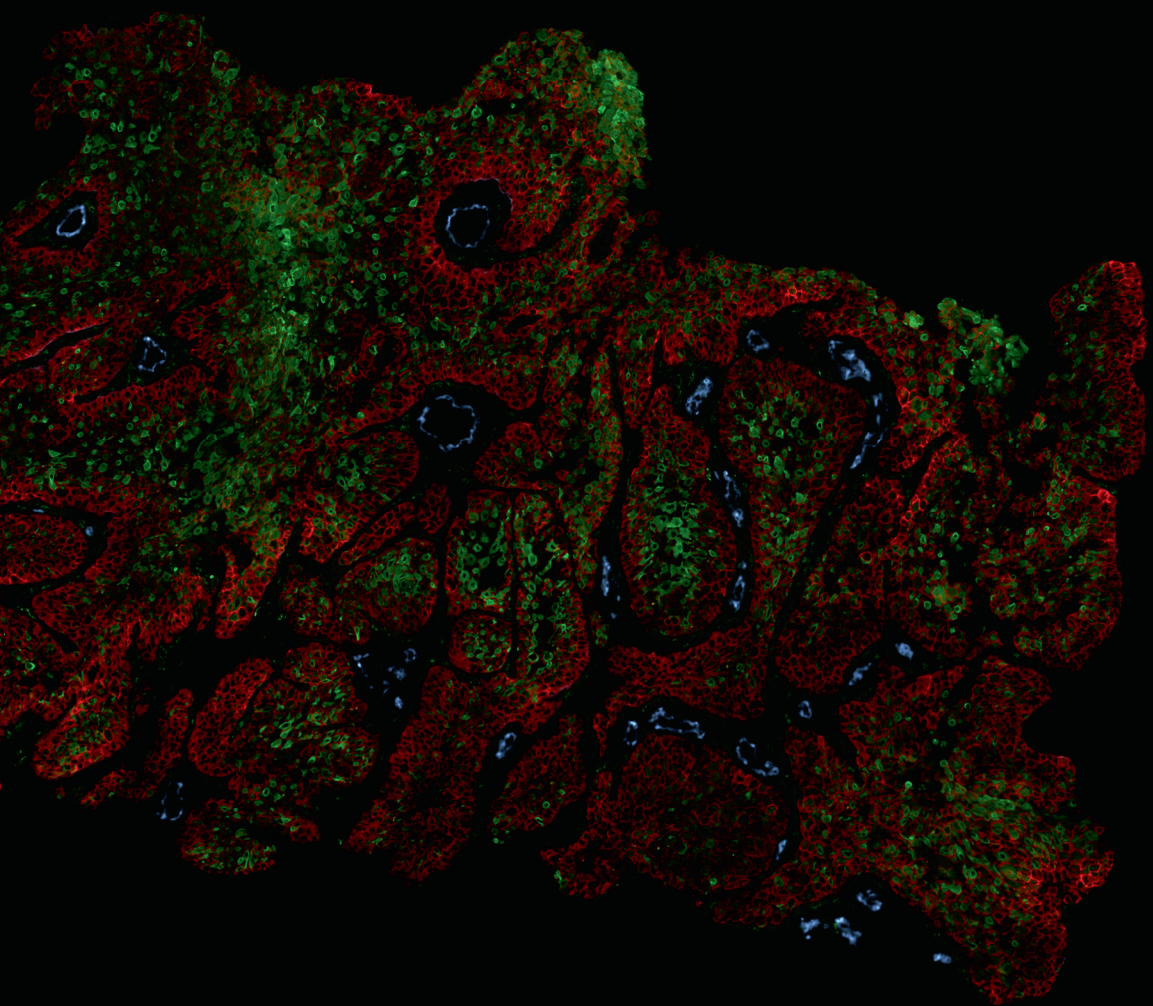
The interaction of the EGFR-PI3-K/AKT pathway with the tumour microenvironment in head and neck cancer



Monique Nijkamp

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Monique M. Nijkamp

Cover J. Lok 2013 Fluorescence image of a head and neck squamous cell carcinoma showing membranous EGFR (red), cytoplasmatic pAKT (green) with vessels (greyblue) continuous in the same section with membranous EGFR, cytoplasmatic pimonidazole (green) with vessels.

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Chapter 1

General introduction and outline of this thesis

The interaction of the EGFR–PI3-K/AKT pathway with the tumour microenvironment in head and neck cancer

Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) belongs to the ten most common carcinomas with an incidence of 800.000 new cases annually worldwide and 2,400 cases annually in The Netherlands. HNSCCs arise from the mucosa of the upper aerodigestive tract and include squamous cell carcinomas of the larynx, pharynx, and the oral cavity [1]. Less frequent tumour entities are tumours of the nasal cavity, paranasal sinuses or salivary gland, and sarcomas. The most common risk factors are alcohol and tobacco abuse, but infection with the Human-Papilloma virus (HPV) has recently been identified as a cause of oropharyngeal cancer in relatively young, non-smoking patients [2]. Successful treatment depends mainly on the stage of the tumour at presentation. Early diagnosis and treatment offers patients the best change of complete recovery. As patients often experience vague complaints such as throat ache or dysphagia, many of them present with advanced local disease already with lymph node metastases [3]. Due to a preference for organ preservation, the principal treatment modality is radiotherapy; which is effective in early-stage tumours, but less effective for advanced tumours and only palliative in metastatic disease [4].

Resistance of tumour cells to radiation is complex and many intrinsic and extrinsic factors are involved. In recent years, research has focussed on optimizing treatment modalities such as accelerated radiation schedules and combination therapies like chemoradiation or molecular targeting agents in addition to radiation to increase survival rates [5,6]. Although only for a selection of patients, (15% at best) there is benefit from these combination therapies, all patients experience the increased side effects. Therefore, it is mandatory to be able to select those patients who are most likely to profit prior to treatment.

Radioresistance mechanisms

The tumour microenvironment plays a key role in resistance to radiotherapy. How well tumour cells respond to radiotherapy does not only depend on their ability to repair the radiation-induced DNA-damage, but also on their proliferative response capacity induced by and during radiotherapy. Furthermore, tumour cell hypoxia induces radioresistance, and in addition, it is also known to promote genetic instability, leading to a more aggressive phenotype with increased tumour cell invasiveness and metastasis, resulting in worse clinical outcome.

Intrinsic radiosensitivity

Although HNSCCs are mostly intermediate sensitive for radiotherapy, there are broad inter-tumour variations. There exist significant patient-to-patient differences between tumours of the same clinical stage and histology in response to radiation treatment. This variation can be explained by differences in intrinsic or microenvironmental factors. Intrinsic radiosensitivity is influenced by factors as the ability to repair radiation-induced DNA single- and double-stranded breaks (DSB). DSBs are potentially lethal DNA lesions and targeting signalling pathways involved in the DNA repair response sensitises tumour cells to ionising radiation. Combining radiotherapy with blockade of these signalling pathways, thereby interfering with DNA DSB repair, has resulted in improved local control compared to radiotherapy alone [7].

Enhanced proliferation

Between treatment intervals, cells are triggered to repopulate more effectively. This enhanced tumour cell proliferation is an important cause of treatment failure [8,9]. Accelerating radiation treatment schedules have been shown to be effective to counteract accelerated proliferation. By delivering more than one fraction per day, the overall treatment time can be reduced whilst maintaining the same total radiation dose. With acceptable acute toxicity levels, shortening the overall treatment time has been demonstrated to contribute to a significant benefit in loco-regional control and disease-specific survival in head and neck cancer patients [10,11]. Tumour cell proliferation depends on multiple factors such as differentiation grade and microenvironmental elements such as oxygen and nutrient availability.

Tumour cell hypoxia

Hypoxic regions are found in almost all solid tumours. Two major forms of hypoxia can be distinguished: chronic (diffusion-limited) and acute (perfusion-limited) hypoxia, although regions of intermediate hypoxia within a tumour can be found (Figure 1). Due to this variable degree of hypoxia, a cut-off value to distinguish normoxic from hypoxic tumour cells does not exist. In most experiments, values below 10 mm Hg have been defined as hypoxic areas although radioresistance may already occur below 25-30 mm Hg [12]. Both forms of hypoxia can co-exist within the same tumour with large differences between tumours regarding the amount of hypoxia and the relative contribution of acute versus chronic hypoxia. Acute hypoxia is often transient and due to a chaotic network of blood vessels with leaky vessels, shunts and other structural and functional abnormalities. Chronic hypoxia is caused by tumour growth thereby increasing the distance of tumour cells to the nearest blood vessels, leaving cells deprived of oxygen and nutrients [13]. There are many studies linking tumour cell hypoxia to the prognosis of head and neck cancer patients and outcome after radiotherapy [14,15]. Because of the “oxygen enhancement ratio (OER)”, hypoxic

cells are approximately three-fold more radioresistant than are normoxic cells [16,17]. Directly, oxygen is essential for the efficacy of radiation-induced DNA damage. Indirectly, hypoxia can lead to activation of hypoxia-induced proteins and genes responsible for tumour progression via various mechanisms [18]. These alterations may help tumour cells to become less dependent of oxygen for survival or help cells to escape to a more favourable environment, for example by the transformation of cells from an epithelial to a more mesenchymal phenotype (EMT) [19]. This leads to more aggressive tumour cells that are able to spread, forming regional and distant metastases and ultimately negatively affecting the prognosis for the patient.

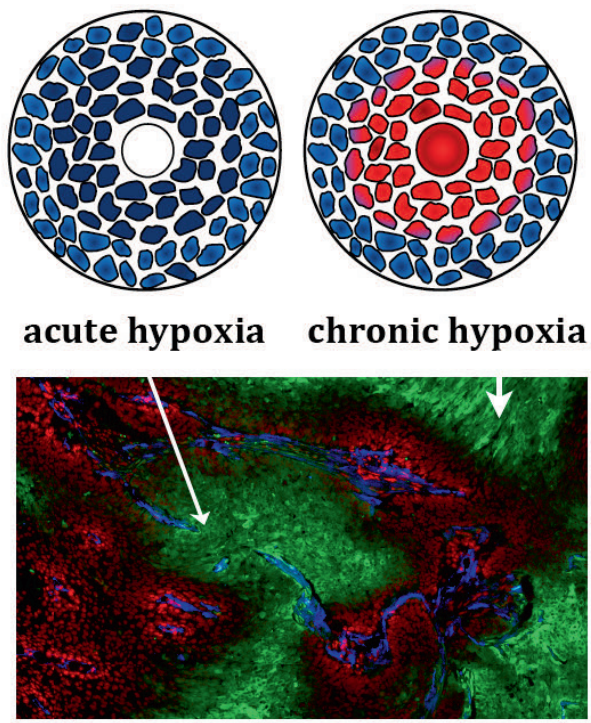

Figure 1. Acute & chronic hypoxia. Acute hypoxia is due to occluded blood vessels while chronic hypoxia is caused by increased distance of tumour cells to the nearest blood vessels. Hypoxia staining by pimonidazole (green), perfusion (red) of blood vessels (blue).

Metastases formation

Metastasis formation not only involves EMT, but also the detachment from the primary tumour site and escape of single cells into the blood or lymph vessels followed by reattachment, transition back to the epithelial state and angiogenesis are required to form a secondary tumour [20,21]. During the first step, EMT, the expression of epithelial markers (for example E-cadherin) is suppressed and the expression of mesenchymal markers (vimentin) is enhanced. Regional and distant metastases have a major impact on prognosis and it is therefore of great importance to understand the mechanisms to be able to explore strategies that interfere early in this process.

Epidermal Growth Factor Receptor

The Epidermal Growth Factor Receptor (EGFR) is a transmembrane protein with intrinsic tyrosine kinase activity and commonly overexpressed in most epithelial cancers in particular HNSCC [22]. Overexpression correlates with resistance of tumour cells against radiotherapy and poor prognosis [23]. Ligand binding to EGFR, for example by EGF, or activation by ionizing radiation [24,25] induces conformational changes leading to receptor homo- or heterodimerization at the plasma membrane with one of its family members, ErbB2 (HER2), ErbB3 or ErbB4. As Figure 2 shows, activation of EGFR causes autophosphorylation (pEGFR) resulting in receptor internalisation and stimulation of many signalling pathways including RAS-mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase-AKT (PI3-K/AKT), signal transducers and activators of transcription (STAT) and the phospholipase C gamma (PLC- γ) pathways [7,24,26]. All these pathways are responsible for regulation of tumour cell proliferation, DNA-damage repair, migration, angiogenesis, and tumour cell survival. The main focus of this thesis will be the activation of the PI3-K/AKT pathway.

PI3-K/AKT pathway

EGFR can lead to activation of the protein AKT by phosphorylation of Thr308 and Ser473 at the cell membrane via activation of PI3K. Phosphorylated AKT (pAKT) then translocates to the cytoplasm and nucleus leading to transcription of genes responsible for tumour progression, such as cellular proliferation and DNA-damage repair. Also genes involved in the cellular response to hypoxia, for example hypoxia-inducible factor1α (HIF-1α) and vascular endothelial growth factor (VEGF) are, at least partly, controlled by pAKT. Besides activation through EGFR, the PI3-K/AKT pathway can be activated by several other mechanisms. These include activation through other receptor tyrosine kinases (RTKs), mutations in oncogenes upstream such as *ras*, loss of Phosphatase and tensin homolog deleted on chromosome 10

(PTEN) [27], or amplifications or mutations of the gene *pik3ca*, encoding the catalytic subunit of PI3K, or AKT itself [28,29].

The EGFR-PI3-K/AKT pathway is relevant for radiation response since it regulates a variety of cellular functions, including proliferation rate, DNA-repair and metastasis formation. In addition, a feedback between hypoxia and EGFR activation exists, making this pathway a key element in treatment responsiveness and therapeutic targeting in head and neck cancer.

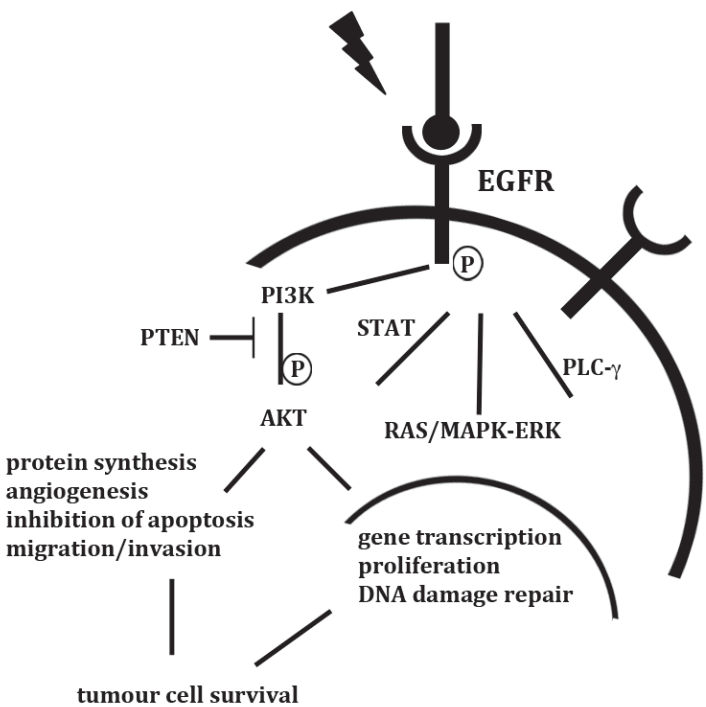

Figure 2. Downstream signalling pathways of the Epidermal Growth Factor Receptor (EGFR). Scheme only denotes a simplified representation.

Prognostic and predictive value of EGFR-PI3-K/AKT signalling

Several investigators have explored the prognostic role of EGFR in solid tumours. Mostly, they found a strong correlation between high EGFR expression and poor clinical outcome and resistance to radiotherapy [23,30]. EGFR activation can lead to enhanced proliferation during radiotherapy. Patients with high EGFR levels showed increased locoregional control rates after treatment with accelerated radiotherapy

compared to conventional radiotherapy schedules, while there was no benefit for those with low EGFR expression [31,32]. Thus, EGFR expression in HNSCC is a predictive factor for a benefit from accelerated radiation treatment. Intensive research during the last decade with focus on EGFR inhibition has resulted in a randomised clinical trial in patients with advanced head and neck cancer reporting an improved loco-regional control and survival when radiotherapy is combined with cetuximab, a monoclonal antibody against the outer part of the EGF receptor [6]. Unfortunately, these improvements are modest whereas all patients experience increased toxicity to some extent. Besides this, although EGFR expression is an important prognostic marker, it does not seem to predict treatment response to anti-EGFR therapy. A possible explanation is that overexpression of EGFR in tumour cells does not necessarily implicate phosphorylation of the receptor and activation of downstream pathways; cells have to be dependent on EGFR signalling for their survival in order to respond to EGFR inhibition.

Next to the prognostic value of EGFR, pAKT was also found to be an independent significant factor for patient outcome. Although most studies correlate high pAKT expression to poor outcome [33-37] there are also studies linking high pAKT levels to better survival [38-41] or reduced migration with variable results in different tumour types [42-45]. These contradictory results point out the need for tumour-type specific research to unravel this complex signalling cascade.

The tumour microenvironment plays an important role in signalling of EGFR and activation of AKT. Not only do not all EGFR expressing cells activate AKT signalling, it is also shown that in hypoxic areas within tumours of the head and neck pAKT is present without EGFR expression. Cells that retain their potential to activate survival pathways in harsh circumstances like hypoxia represent an important subpopulation of tumour cells that are responsible for treatment failure [46]. Besides immunohistochemically staining for EGFR and pAKT expression, tumour cell hypoxia and proliferation can be assessed in patient biopsies prior to treatment. Endogenous markers such as Ki-67 and proliferating cell nuclear antigen (PCNA) or exogenous markers like S-phase specific thymidine analogues bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd) are available to indicate the amount of proliferating tumour cells. In several studies the relationship between these markers and response to radiotherapy was assessed, with inconclusive results about their predictive value [31,47,48]. This suggests that the proliferative response of tumours during treatment is probably independent of baseline proliferation. Hypoxic areas can be stained by intravenously administered bio-reductive chemical markers such as the nitromidazoles pimonidazole or EF5 [49]. These markers have the advantage that they only metabolise in active cells and, therefore, necrotic tissue does not generate a

signal. Endogenous hypoxia-related markers are involved in the tumour cell response to hypoxia, but also to other stress-related aspects like intratumour pH. Proteins that show the most promising results as potential hypoxic markers are hypoxia-inducible factor 1α (HIF-1α), carbonic anhydrase IX (CAIX) and glucose transporters 1 and 3 (GLUT) [13,50].

Counteracting radioresistance in head and neck cancer

To overcome enhanced tumour cell proliferation during treatment and tumour cell hypoxia numerous strategies have been clinically tested. Different studies show that accelerated tumour cell proliferation can be counteracted by accelerated radiotherapy [10,11], while tumour hypoxia can be reduced using a hypoxia-modifying treatment combined with radiotherapy [51,52]. These latter treatments vary from hyperbaric oxygen and carbogen breathing to hypoxic cell sensitizers, using nitroimidazoles, and hypoxic cytotoxins, to destroy hypoxic cells [53]. A recently published meta-analysis including 32 randomized clinical trials demonstrated that there is a significant beneficial effect of hypoxic modification if combined with radiotherapy [54].

A strategy that integrates both accelerated radiotherapy and hypoxia modification is **A**ccelerated **R**adiotherapy with **C**arb**O**gen and **N**icotinamide (ARCON) [16]. Breathing carbogen, a hyperoxic gas (98% O_2; 2% CO_2), during accelerated radiation treatment will sensitize tumour cells through reduction of diffusion-limited hypoxia. The orally administered vasodilating compound nicotinamide reduces perfusion-limited hypoxia by preventing intermittent closure of tumour blood vessels. A phase II study involving 215 patients with advanced head and neck cancer showed high local and regional control rates, in particular for oropharynx and larynx tumours [55]. Results from a subsequent phase III study involving 345 patients with laryngeal carcinoma showed higher regional control rates without an increase in toxicity [56]. Results from a side study using the exogenous hypoxia marker pimonidazole revealed that this increase was only significant in the more hypoxic tumours. Patients with hypoxia higher than median levels receiving ARCON had higher regional control and disease-free survival rates compared to the standard AR treatment. Patients with well-oxygenated tumours did not benefit from the addition of carbogen and nicotinimide to radiotherapy. This illustrates that identification of microenvironmental characteristics, in this example the amount of hypoxia, may allow a better selection of patients for different treatment methods, with the ultimate aim to provide the best quality of life for individual cancer patients.

Another way to try to predict patients from a beneficial effect of ARCON treatment is possibly via the activation of the EGFR-PI3-K/AKT pathway, which is associated with proliferation and hypoxia response. This signalling pathway may influence the therapeutic effect and may therefore be a powerful predictor for treatment with ARCON.

Outline of this thesis

The central aim of the present thesis is to investigate the activation of the EGFR-PI3-K/AKT pathway in biopsies of head and neck squamous cell carcinoma patients and its implication for radiation resistance. Besides their potential prognostic or predictive value, we evaluated their expression related to microenvironmental factors, like the presence of hypoxia or vasculature.

Chapter 2 gives an overview of the role of EGFR in head and neck cancer, its interaction with the tumour microenvironment and their involvement in radioresistance. This chapter illustrates that both irradiation and hypoxia can influence EGFR activation and we therefore hypothesized that EGFR could modulate the response to accelerated radiotherapy and hypoxia modification. **Chapter 3** describes the findings on the predictive value of EGFR expression in 272 laryngeal cancer patients on the outcome of hypoxia modification in addition to accelerated radiotherapy (ARCON).

In **chapter 4**, the relative contribution of activated EGFR on the activation of the PI3-K/AKT pathway was examined in a cohort of 58 patients. We investigated expression levels of pEGFR and pAKT and correlated these to patient outcome. Also, the relationship between pEGFR, pAKT, vessels, and hypoxia was assessed. We discovered a distinct relationship of pAKT expression in the primary tumour of HNSCC patients and lymph node metastases. Our hypothesis was that this counterintuitive correlation between pAKT and an increased metastasic risk might be due to epithelial-mesenchymal transition (EMT). This led us to explore the association of markers; i.e. E-cadherin and vimentin, involved in EMT in this patient group.

Chapter 5 describes the association between EGFR and pAKT and E-cadherin and vimentin expression. Low E-cadherin and high vimentin expression lead to reduced cell-cell contact and higher possibility of cells to migrate and form metastases.

There is an increasing number of early clinical trials exploiting the inhibition of specific target proteins and genes in addition to radiotherapy. One such inhibitor is MK-2206, an allosteric inhibitor of pAKT. It alters the shape of AKT so that ATP binding at the phosporylation sites Th308 and Ser473 is prevented. Due to our previous finding that pAKT expression seemed to be involved in increased regional and distant metastatic risk we performed experiments to investigate the effect of pAKT inhibition on EMT in laryngeal cancer cells, which are described in **chapter 6.**

Chapter 7 provides a general discussion including future perspectives. A summary of this work is given in **chapters 8** (Dutch) and **9** (English).

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Chapter 2

Interaction of EGFR with the tumour microenvironment: implications for radiation treatment

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Paul N. Span
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Radiotherapy & Oncology 2013 Jul;108(1):17-23

Abstract

Treatment failure through radioresistance of tumours is associated with activation of the epidermal growth factor receptor (EGFR). Tumour cell proliferation, DNA-repair, hypoxia and metastases-formation are four mechanisms in which EGFR signalling has an important role. In clinical trials, a correlation has been demonstrated between high EGFR expression in tumours and poor outcome after radiotherapy. Inhibition of EGFR signalling pathways improves the effectiveness of radiotherapy of head and neck cancers by overcoming these main mechanisms of radioresistance. The fact that only a minority of the patients respond to EGFR inhibitors reflects the complexity of interactions between EGFR-dependent signalling pathways and the tumour microenvironment. Furthermore, many components of the microenvironment are potential targets for therapeutic interventions. Characterisation of the interaction of EGFR signalling and the tumour microenvironment is therefore necessary to improve the effectiveness of combined modality treatment with radiotherapy and targeted agents. Here, the current status of knowledge is reviewed and directions for future research are discussed.

Introduction

The epidermal growth factor receptor (EGFR) attenuates the efficacy of radiotherapy in tumour cell killing through its association with enhanced proliferation, DNA-repair and hypoxia. Intracellular signalling of EGFR occurs via phosphorylation cascades in different pathways in which protein kinase B (PKB/AKT) is a key-regulating factor. However, immunohistochemical staining of EGFR and phosphorylated (p)AKT shows that EGFR-independent activation of AKT also occurs, predominantly in hypoxic areas [1,2]. This observation suggests that activation of specific proteins in the important signalling cascades may also depend on microenvironmental characteristics, such as tumour oxygenation status. Vice versa, manipulation of EGFR affects the tumour cells [1]. Understanding the microenvironmental conditions that influence important signalling pathways in tumours insensitive to particular treatment regimens can improve selection of patients for individualized treatment options. In this review we will discuss the interactions between the EGFR signalling cascade and the tumour microenvironment (Figure 1), using mainly preclinical data as well as the available, albeit limited information from clinical studies.

Tumour microenvironment

Our current conception of a malignant tumour is that of a complex structure containing not only cancer cells but also a variety of normal cell types that intimately interact with a microenvironment that is characterised by both temporal and spatial heterogeneity. It has become clear that this tumour microenvironment is important during early cancer development and progression, and is also of influence on the response of tumours to radiation [3,4]. Elements that make up the tumour microenvironment include endothelial cells of the blood and lymphatic vessels, fibroblasts, infiltrating cells of the immune system and the tumour extracellular matrix (ECM) [5]. Availability of oxygen and nutrients depends on the functionality of the vascular bed and affects the metabolic state of tumour and stromal cells. An imbalance between oxygen and glucose supply and consumption will result in hypoxia and acidification. Within the tumour microenvironment, hypoxia is relevant in almost all solid tumours. Reduced oxygen supply can be lethal for some cells, but others are able to survive under even severe or prolonged hypoxic conditions. Hypoxia-induced cell signalling promotes tumour growth, migration and survival. The development of new vasculature within a tumour involves the formation of new vessels from endothelial cells (vasculogenesis) in addition to sprouting (angiogenesis) of new vessels from existing ones [6]. New tumour blood vessels, prerequisite for tumour progression and metastasis formation, the result of interplay between pro- and anti-angiogenic factors, is predominantly regulated through transcription of the hypoxia-inducible-factor (HIF)-1 complex. The pro-angiogenic factor VEGF is a crucial gene involved in angiogenesis that is strongly induced by hypoxia. Anti-angiogenic therapy,

for example by using anti-VEGF monoclonal antibodies such as bevacizumab, can result in a reduction of tumour vascularisation, but counter-intuitively also in normalisation of the aberrant tumour vasculature, thereby improving oxygenation and blood flow that could enhance the efficacy of radiation [7,8]. However, VEGF can also be upregulated in an oxygen-independent manner. Also, low extracellular pH causes stress-induced alteration of gene expression including the upregulation of VEGF in tumour cells *in vitro*. Tissue pH appears to regulate VEGF transcription through a different pathway independently of hypoxia, namely Ras-ERK1/2 instead of HIF mediated [8].

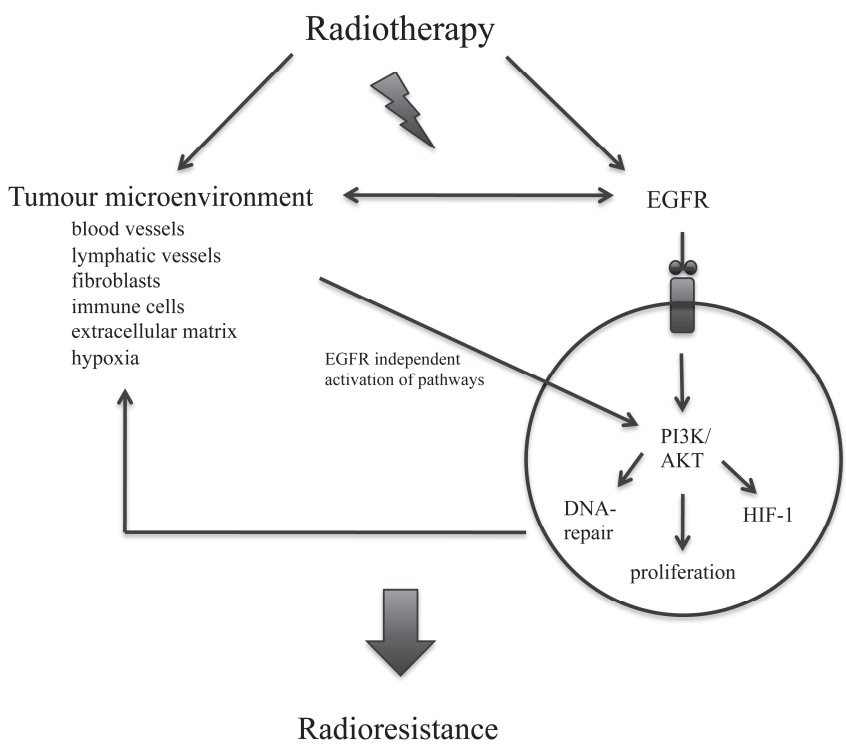

Figure 1. Interaction between EGFR signalling, radiotherapy and the tumour microenvironment leading to radioresistance. Different aspects of the tumour microenvironment can activate signalling pathways, via EGFR or directly and EGFR-independent. Activation of downstream proteins/genes affects the tumour microenvironment, e.g. via angiogenic factors and effects on the extracellular matrix. Both tumour microenvironmental factors as well as EGFR signalling can lead to more radioresistant tumours.

Cancer-associated fibroblasts can influence different aspects of tumour cell behaviour such as growth and migration trough release of growth factors and chemokines. Various cells of the immune system found in solid tumours play an important role in modulating tumour growth. Macrophages form a major inflammatory population in most cancers but other components of the inflammatory infiltrate also module tumour behaviour, having pro- and anti-tumour functions [5].
Exchange of information between tumour cells can occur from the ECM to tumour cells directly, via mechanical forces, or can be mediated by ECM-associated growth factors [9]. The signals triggered by components of the ECM are not function-specific and depending on the local environment they can induce proliferation as well as the phenomen known as epithelial-mesenchymal transition (EMT). During this process tumour cells change from an epithelial morphology to a migratory and invasive, mesenchymal phenotype [10]. Growth factors, such as EGF, and stress stimuli like hypoxia, have been shown to induce EMT *in vitro* by inducing phosphorylation of E-cadherin resulting in its degradation thereby linking growth factor receptor signalling to the induction of EMT [11].

Epidermal growth factor receptor and downstream signalling pathways

EGFR is a transmembrane protein with intrinsic tyrosine kinase activity that is overexpressed in most epithelial cancers, e.g. in over 80% of head and neck squamous cell carcinomas (HNSCC) [12,13]. Overexpression could lead to resistance of tumour cells against radiation as demonstrated by *in vivo* studies [14-16] and is associated with poor prognosis in HNSCC [13]. (p)EGFR and HER2 expression are mostly determined by intrinsic features of the tumour cell, while the activation of downstream kinases is highly influenced by the tumour microenvironment [17]. Ligand binding to EGFR induces conformational changes leading to receptor homo- or heterodimerization at the plasma membrane with one of its family members, ErbB2 (HER2), ErbB3 or ErbB4. This causes autophosporylation, subsequent receptor internalisation and stimulation of multiple signalling pathways including ras-mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase-AKT (PI3-K/AKT)), signal transducers and activators of transcription (STAT) and the phospholipase C gamma (PLC- γ) pathways [3,18,19]. These pathways all share that they counteract radiation efficacy being involved in proliferation, migration, apoptosis and angiogenesis. Of note is that, besides through ligand binding, EGFR can also be activated by ionizing radiation itself, again leading to radioresistance [18,20-22]. EGFR tyrosine kinase inhibition with erlotinib or gefitinib improves progression-free survival in advanced non-small cell lung cancer (NSCLC) with EGFR mutations [23]. In preclinical studies EGFR-expression was needed for C225-response, but this was not sufficient to predict response to C225 plus radiotherapy. Evaluation of the microenvironment revealed that basal expression of

additional growth factor receptors and effects on proliferation, correlated to a certain extend with response to combined C225-radiotherapy [24]. Combining radiotherapy with blockage of EGFR by the chimeric (mouse/human) monoclonal antibody cetuximab, has resulted in improved locoregional control and survival for patients with HNSCC [25] demonstrating that EGFR is an clinically relevant target for molecular therapies in addition to radiation.

A key protein activated through EGFR is AKT that can be phosphorylated at Thr308 and Ser473 at the cell membrane after activation of PI3-K. pAKT then translocates to the cytoplasm and nucleus where it can activate or deactivate a myriad of substrates via its kinase activity or via transcription of genes responsible for tumour progression. Although most studies correlate high pAKT expression to poor local control [26,27], there are also studies linking high pAKT to better survival or reduced migration [2,28]. This suggests that the concept of EGFR induced AKT activation leading to treatment resistance and poor outcome is a simplification of a complex interaction between the EGFR signalling network and the tumour microenvironment. Importantly, it is shown that hypoxia can induce cellular changes and in hypoxic areas of HNSCC activated AKT has been observed in absence of EGFR expression (Figure 2). Immunohistochemical staining for EGFR and pAKT in biopsies of patients with HNSCC reveals a lack of association: tumour cells positive for EGFR were found negative for pAKT and vice-versa (Figure 3). Although a better correlation between activated EGFR (pEGFR) and pAKT existed, there were still tumour cells present with pAKT but no pEGFR [2]. A possible explanation is that AKT can be activated by different members of the ErbB family and other type of receptors like VEGFR. This is supported by the observation that blocking VEGFR-2 caused a suppression of pAKT [29]. Also, these observations suggest that the tumour microenvironment may stimulate activation of AKT in an EGFR-independent manner and is likely to be involved in the other EGFR-driven signalling pathways as well.

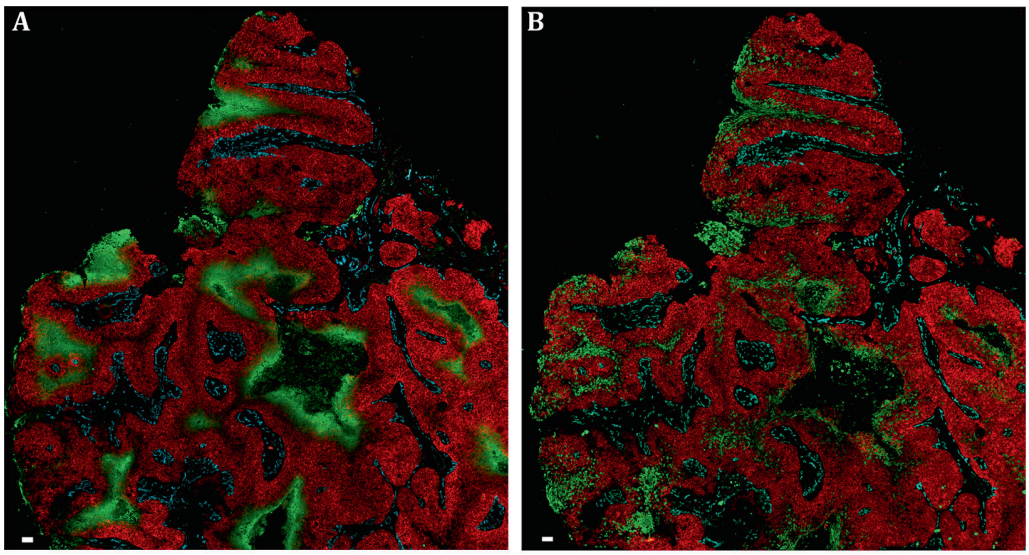

Figure 2. Immunohistochemial staining of whole consecutive laryngeal tumour sections shows that pAKT (green) expression (B) occurs predominantly in hypoxic areas stained with pimonidazole (green; A). Red is EGFR expression, mostly seen in normoxic areas closer to tumour blood vessels (white). 200X magnification. Scalebars represent 100 μm.

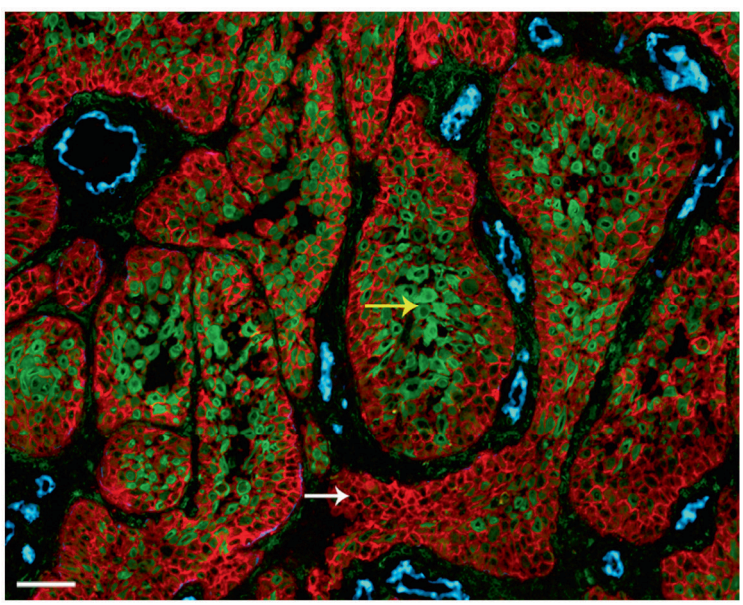

Figure 3. Mismatch of EGFR (red) and pAKT (green) in tumour sections of a laryngeal carcinoma (200X magnification). A Tumour section with EGFR expression present but no activated AKT (white arrow) and tumour cells with EGFR-independent pAKT expression (yellow arrow). White is tumour vasculature (stained with PAL-E). Scalebar represents 100 μm.

Tumour microenvironmental factors and radioresistance

Not only do tumours from different origins respond differently to radiotherapy, also tumours with similar pathology show broad variations in response. Resistance of cancer cells to radiation is complex and many intrinsic and extrinsic factors are involved. Activation of EGFR signalling pathways can influence various cellular functions that are involved in the major mechanisms leading to radioresistance including tumour cell proliferation, DNA-repair and hypoxia. Activation of EGFR is also involved in a fourth mechanisms leading to radioresistance of tumours namely the escape of tumour cells from local therapy by radiation through the formation of metastases.

Tumour cell proliferation

The accelerated proliferation rate of tumour cells during radiotherapy is one reason for locoregional failure [30]. A number of clinical trials have confirmed that shortening the overall treatment time leads to increased tumour control probability by reducing the possibility of tumour cells to enhance repopulation between radiotherapy fractions [31]. This accelerated proliferation rate can be a result of activation of EGFR in response to ionizing radiation and an explanation why tumours with high EGFR expression have a worse prognosis [13]. Two clinical studies have indeed confirmed that accelerated radiotherapy, either moderate acceleration with 6 fractions per week over 5.5 weeks or accelerated hyperfractionation with 3 fractions a day, and a total treatment time of 12 consecutive days, results in better locoregional control when EGFR was overexpressed, but not in tumours with low EGFR expression [30,32]. This suggests that EGFR-related signalling is involved in the proliferative response to radiotherapy thereby enhancing tumour survival probabilities. Preclinical data confirm that radiation-induced activation of EGFR represents a critical step in the activation this mechanism [33]. Large-scale studies with fractionated irradiation in xenografted FaDu (HNSCC) tumours demonstrated that after 3-4 weeks of fractionated radiotherapy an enhancement of repopulation occurs. At the same time an upregulation of EGFR expression was observed, indicating that EGFR is involved in this response [34]. Inhibition of EGFR through cetuximab [35] or tyrosine-kinase inhibitors such as erlotinib [15] was found to reduce tumour cell repopulation. EGFR inhibition with cetuximab during fractionated radiotherapy reduced tumour cell repopulation and improved local control in FaDu tumours [35] but also leads to a change in spatial distribution of EGFR favouring the membranous expression [36]. Erlotinib can inhibit radiation-induced activation of EGFR thereby reducing its proliferative signalling capacity. Both agents have demonstrated to lower the percentage of human tumour cells in the more radioresistant S-phase fraction and induce an accumulation of cells in the more radiosensitive cell cycle phases [37].

Consistent with these findings, Krause et al. observed a significantly lower S-phase fraction measured by BrdU labelling after treatment with a tyrosine kinase inhibitor in FaDu tumours compared to untreated tumour. However, this reduced proliferation did not lead to improved local tumour control after radiation [38]. Additionally, Gurtner et al showed that in a panel of HNSCC models erlotinib as well as cetuximab lead to tumour growth delay but only simultaneous application of cetuximab during fractionated irradiation improved local control, while erlotinib did not enhance the radiotherapy effect [39], illustrating that different models of EGFR inhibition may lead to different ultimate treatment results.

DNA-repair

Activation of the EGFR downstream pathways RAS and PI3-K/AKT have been found to increase the resistance of tumour cells to agents that cause DNA damage [40]. DNA double strand breaks (DSB) are the most important DNA lesions leading to cell kill after radiotherapy. Tumour cells can repair DSBs through non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the major process responsible for survival of cells exposed to ionizing radiation, making this type of repair probably most influencial for treatment outcome. An important molecular complex involved in this process is the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which is known to reside primarily in the nucleus. An interaction between nuclear EGFR and DNA-PKcs has been described suggesting a crucial role of nuclear EGFR for regulation of DNA repair after radiation [41]. Activated EGFR can be internalized and translocated to the nucleus and irradiation-induced nuclear EGFR can stimulate the formation of the nuclear EGFR/DNA-PKcs complex [42]. Selective inhibition of these pathways was found effective in reducing tumour cell survival. For example, cetuximab blocked radiation-induced nuclear translocation of EGFR and was associated with inhibition of radiation-induced activation of DNA-PKcs in a human bronchial carcinoma cell line [42]. Combined treatment of radiation and cetuximab resulted in a redistribution of DNA-PKcs from the nucleus to the cytoplasm. The reduction of DNA-PKcs in the nucleus leads to impeded NHEJ, essential for DNA-repair and survival. This might be a potential mechanism of the combined modality approach whereby the repair of DNA-DSBs after radiotherapy is impaired by cetuximab [37]. Blockage of radiation-induced DNA-PKcs activation by EGFR, PI3-K or AKT inhibition as well as through knockdown of AKT1 by siRNA indicates the requirement of the EGFR-PI3-K/AKT pathway for regulation of DNA-DSB repair after exposure to ionizing radiation [43]. Selective EGFR inhibition by gefitinib increased radiosensitivity of stem-like gliomaspheres by reducing DNA-PKcs expression, accompanied by reduced repair of radiation-induced DNA DSBs [44]. These studies suggest that EGFR mediated repair of DNA damage might play a prominent role in the mechanism of radioresistance. Further preclinical *in vivo* and clinical research is required to

determine to what extent this is a tumour type specific mechanism and to identify predictors of response to EGFR- or downstream PI3-K/AKT-targeted DNA-repair inhibitor.
In conflict with other studies, a novel finding showed that cetuximab as well as irradiation can promote EGFR translocation to the nucleus. Cetuximab treatment resulted in phosphorylation of the $EGFR^{y845}$ site leading to an increased translocation to the nucleus of HNSCC tumour cells. This process can be inhibited by dasatinib, an inhibitor of several kinases including SRC family kinases (SFKs), which are suggested to be necessary for the phosphorylation of the $EGFR^{y845}$ site and cetuximab-induced EGFR translocation to the nucleus [45]. Whether this cetuximab-induced nuclear EGFR is able to activate target genes to the same extend as ligand binding or radiation does is still unclear.

Hypoxia
The consequences of tumour cell hypoxia for treatment and patient outcome have been well established [46,47]. Hypoxia is associated with treatment failure as hypoxic tumour cells are significantly more resistant to radiation than normoxic cells. Although hypoxia is considered a limiting factor for tumour growth it is a stimulus for invasion and metastasis formation [5]. To adapt to hypoxic conditions, cells can respond by activating hypoxia-inducible genes or pro-survival signalling pathways, directly or indirectly through induction of the transcription factor hypoxia-inducible-factor (HIF)-1 complex [48,49]. HIF-1 modulates the expression of genes involved in cell survival, angiogenesis and migration [10,50,51]. After HIF activation by hypoxia, processes are induced in cells to adapt to low oxygen levels, including metabolic changes and angiogenesis. Various treatment strategies have been developed to address the hypoxia problem [52,53]. The hypoxic sensitizer nimorazole has been shown to improve locoregional control in HNSCC when applied in conjunction with radiotherapy [54]. Also, ARCON (accelerated radiotherapy with carbogen breathing (98% O_2, 2% CO_2) and nicotinamide) a treatment method that aims to counteract both tumour cell repopulation and hypoxic radioresistance has demonstrated benefit for hypoxic laryngeal carcinomas [55,56]. A meta-analysis demonstrated that there is a level 1a evidence in favour of adding hypoxic modification to radiotherapy in HNSCC [53].
In biopsies of breast cancer patients, expression of HIF-1α is associated with EGFR expression [57]. A feedback loop between hypoxia-induced upregulation of HIF-1α and EGFR provides sustained signalling when oxygenation of tumour cells improves, even up to normoxic conditions. Preclinical research shows that activation of HIF is required for the up-regulation of EGFR protein levels in hypoxic cancer cells. Conversely, EGFR-driven PI3-K/AKT activation in breast cancer cells can also lead to increased levels of HIF-1α independently of hypoxia [58]. The exact mechanism by which PI3-K/AKT signalling mediates the induction of HIF is not clear yet and is not

confirmed in other tumour cell lines [59], suggesting that this phenomenon may be cell type specific. In cervix carcinoma and pheochromocytoma cells, hypoxia resulted in the induction of HIF-1α proteins with AKT activation present. However, after growth factor stimulation under normoxic conditions there was no induction of the HIF protein and/or its transcriptional activity although the treatment resulted in activation of the PI3-K/AKT pathway. These data suggest that merely the activation of this pathway may not be sufficient for the accumulation of the HIF-1α protein, at least not in all tumour types.

Thus, interactions between the EGFR-PI3-K/AKT and HIF pathways vary with tumour type and oxygenation status. Furthermore, different effects can be measured at protein and mRNA levels. For example, blocking EGFR in HNSCC cells leads to decreased translation of HIF-1α protein under hypoxia, but inhibition does not completely eliminate HIF expression under hypoxic circumstances nor are the HIF-1α mRNA levels altered [60]. Pore et al. also found that nelvavir, a drug known to inhibit PI3-K/AKT signalling, decreases HIF-1α protein expression in HNSCC and lung cancer cells indicating that hypoxia-driven EGFR signalling might act via this downstream pathway in these tumour types [61]. HIF activation can also occur via radiation-induced EGFR signalling. Recently, Lu et al. showed that cetuximab could inhibit radiation-induced HIF-1α upregulation in HNSCC [62]. This inhibitory effect of cetuximab was much weaker for hypoxia-induced HIF-1α than for radiation-induced HIF-1α expression supporting the mediator role of EGFR in the latter. In addition, a connection between EGFR and VEGF, a downstream target of HIF-1α exists and EGFR inhibition using erlotinib leads to downregulation of HIF-1α expression and decreased VEGF secretion [29]. Erlotinib improves tumour oxygenation via improved vascular perfusion but this decrease in hypoxia did not seem to have an effect in radiosensitivity in HNSCC xenografts and cells [63]. Gefitinib treatment reduced pimonidazole binding in A431 xenografts after 5 and 8 days of treatment showing that gefitinib reduces intratumoural hypoxia [64].

These data indicate that activation of hypoxia-inducible genes is cell type specific, and that there is an intricate interaction between growth factor receptor activation and microenvironmental signalling. Tumour cell hypoxia can result in creating an optimal environment for tumour regrowth by activating hypoxia-induced genes leading to angiogenesis, while it also is associated with decreased radiation-induced DNA damage and a poorer response to radiotherapy making hypoxia a key element in the clinical outcome of patients [65]. Therefore, inhibition of EGFR in combination with hypoxia modification should be further explored as it might offer a powerful strategy for treatment of a number of cancer types.

Experimental evidence has also provided a relationship between EGFR signalling and angiogenic proteins such as VEGF. Tumours often express high levels of VEGF leading

to an abnormal vasculature. Vascular normalization and an increase in tumour blood flow can be achieved by directly targeting VEGF or its receptor (VEGFR), with for example the anti-VEGFR monoclonal antibody bevacizumab. Also, EGFR inhibition with erlotinib in mice bearing SQ20B head and neck xenografts caused changes in vessel morphology, a decreased vascular permeability and an increase in tumour blood flow. This indicates that EGFR inhibition has an effect on vasculature resulting from a decrease in VEGF expression [63]. The combination of four weeks treatment with cetuximab and ZD6474, a potent inhibitor of VEGFR-2 tyrosine kinase with a significant anti-EGFR tyrosine kinase activity, showed a more significant growth inhibition in mice bearing colon or lung adenocarcinoma xenografts as compared to single agent treatment [29]. A normal tumour vasculature and improvement in tumour blood flow can lead to better drug delivery or increased tumour oxygenation, and, thereby, a better response to radiotherapy.

Metastases formation

The escape of tumour cells from the primary tumour to distant sites and subsequent formation of metastases is a fourth mechanism leading to treatment failure. During EMT, the loss of E-cadherin proteins leads to the disruption of stable adherent junctions. In addition, cells develop a more mesenchymal phenotype, e.g. increased expression of mesenchymal proteins like vimentin, and thereby more able to spread to secondary locations in the body. Increasing evidence indicates that EGFR signalling pathways are implicated in the regulation of proteins involved in EMT. In cervical as well as prostate cancer cells lines, EGF treatment significantly decreased the abundance of E-cadherin protein and upregulated vimentin expression [66,67]. Also, in surgical biopsies of cervical carcinomas EGFR overexpression was accompanied by decreased E-cadherin and increased vimentin expression seen by immunofluorescent staining [66].

More evidence was provided by studies using EGFR inhibitors. An HNSCC tumour model in which cells dominantly express epithelial markers was found to be very sensitive to cetuximab, whereas those expressing mesenchymal markers revealed low sensitivity [68]. Although the exact interaction was not elucidated, this response to EGFR inhibition suggests that there is an association between EGFR pathways and EMT. However, this interaction between EGFR signalling and EMT seems to be tumour line specific. Combining cetuximab with irradiation induced EMT in the cetuximab-sensitive cells while triggering the reverse mesenchymal-epithelial transition (MET) in the more mesenchymal cell line [68]. The idea is that cells that have undergone EMT become less dependent on EGFR signalling for cell proliferation and survival and are thus less responsive to EGFR inhibitors [69]. This also points towards a possibility of using EMT-related proteins as predictive markers for sensitivity to cetuximab. This hypothesis needs to be confirmed in tumours from patients either sensitive or resistant to EGFR inhibition but it clearly suggests a potential for individualised

treatment approaches employing EGFR-targeting or more aggressive anti-metastasis treatment based on EMT phenotype.

Conversely, it has been shown that E-cadherin-mediated-cell-cell adhesion can trigger a ligand-independent activation of EGFR thereby regulating various signalling pathways such as MAPK and AKT [70,71]. Reddy et al. showed that activation of AKT and MAPK by E-cadherin mediated cell-cell adhesion in ovarian cancer cells is regulated by EGFR activation. However, no direct physical interaction between E-cadherin and EGFR could be detected in the cells used, suggesting the existence of intermediate molecules [71]. In contrast, it was demonstrated that in oral squamous carcinoma cells E-cadherin can physically interact with and activate EGFR, leading to the activation of MAPK [70]. This interaction leads to EGFR-E-cadherin complex formation at cell-cell junctions and receptor oligomerization. Although the exact mechanism is unclear and needs to be verified, both studies show that E-cadherin may not only act as an adhesion molecule but also as an upstream regulator that triggers EGFR signalling pathways.

Further, a relation between oxygenation status and EMT also exist. Hypoxia can down regulate E-cadherin and upregulate mesenchymal markers, indicating that hypoxia can contribute to metastases-formation through induction of EMT [72]. The morphological transformation induced by hypoxia in breast cancer and FaDu cells associated with EMT can be reversed after re-oxygenation or by repression of HIF-1α [72,73]. This finding at least partly explains the relation between tumour cell hypoxia, migration and ultimately metastasis formation.

Conclusion

There is strong evidence, both from preclinical and clinical studies that there is a positive correlation between the levels of EGFR found in tumour cells and resistance to radiation therapy and consequently treatment failure. EGFR signalling pathways are implicated in all major mechanisms of radioresistance. The tumour microenvironment has important influences on EGFR signalling. The fact that only a minority of the patients respond to EGFR inhibitors reflects the complexity of interactions between the EGFR-dependent signalling pathways and the tumour microenvironment. To improve the effectiveness of combined modality treatment with radiotherapy and targeted agents two strategies should be explored. One is patient selection based, not only on EGFR expression patterns, but also on microenvironmental characteristics to identify the tumour phenotypes that are most likely to benefit from the combined approach. Second is to combine radiotherapy, not only with EGFR signalling inhibition but also with treatments that counteract microenvironmental resistance mechanisms such as hypoxia, e.g. nimorazole, ARCON or angionesesis inhibition (VEGF inhibitors). Future preclinical and clinical studies

should focus on these multimodality approaches with mechanistic basis to bring cancer research forward.

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Chapter 3

Epidermal growth factor receptor expression in laryngeal cancer predicts the effect of hypoxia modification as an additive to accelerated radiotherapy in a randomised controlled trial

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Abstract

Accelerated radiotherapy (AR) improves the poor prognosis associated with Epidermal Growth Factor Receptor (EGFR) overexpression frequently seen in head and neck carcinomas. Combining AR with carbogen and nicotinamide (ARCON) counteracts enhanced tumour cell proliferation- and hypoxia-related radioresistance. The purpose of this study was to investigate if EGFR expression levels are associated with response to ARCON in patients with carcinoma of the larynx.

Patients (N=272) with advanced stage larynx carcinoma were randomized between AR alone and ARCON. Paraffin-embedded biopsies from these patients were processed for immunohistochemical staining of EGFR. EGFR fraction was quantitated by automated image analysis and related to clinical outcome.

A large variation was observed in EGFR fraction between tumours with expression levels ranging from 0-0.93 (median fraction 0.4). No difference in 5-year locoregional control was found between low and high EGFR expressing tumours in the AR arm (69% versus 75%), which is in line with the established effect of AR in EGFR overexpressing tumours. There was, however, a significant association in the ARCON arm: patients with low EGFR levels had a better 5-year locoregional control (88% versus 72% p=0.02) and disease-specific survival (92% versus 77% p=0.01). ARCON improved locoregional control relative to AR only in patients with low EGFR expression (HR 0.34 p=0.009).

In conclusion, only in tumours with a low EGFR fraction, adding hypoxia modification to AR has an additive beneficial effect on outcome. EGFR expression is a predictive biomarker for the selection of patients that will or will not respond to ARCON.

Introduction

The Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor tyrosine kinase that plays a major role in regulating tumour cell proliferation and cell cycle progression [1,2]. EGFR is highly expressed in many solid cancers, including head and neck squamous cell carcinomas (HNSCC) [3] and is correlated with resistance to radiotherapy and decreased patient survival [4]. Ligand binding as well as ionizing radiation can phosphorylate EGFR [5] leading to activation of downstream cascades, like the PI3-K/AKT and the MAPK pathways [6]. These signalling pathways are responsible for enhanced proliferation, cell cycle progression and increased DNA-repair leading to treatment failure [2,7,8].

How well tumour cells respond to radiotherapy depends on their proliferative response induced by and during treatment, their ability to repair the radiation-induced DNA-damage and the amount of hypoxia within a tumour [9]. EGFR is involved in the regulation of intrinsic DNA-repair mechanisms and tumour cell proliferation via downstream pathway activation [10]. Tumour cell hypoxia induces radioresistance, directly as DNA-damage is maximized in the presence of oxygen and indirectly by promoting genetic instability [11,12]. An autocrine route has been described by which hypoxia induces expression of EGFR and its ligands [13,14] and in addition, EGFR can stabilize one of the key proteins in the hypoxia response namely hypoxia-inducible factor 1α (HIF-1α) [11]. Thus, EGFR is involved in all aspects of radioresistance. These resistance mechanisms play a role in HNSCC while EGFR is expressed at high levels in the majority of these tumours. This makes head and neck cancer the tumour archetype to further investigate these interactions.

Various studies have shown that enhanced tumour cell proliferation can be counteracted by accelerated radiotherapy (AR) [15-17], while tumour hypoxia can be reduced using hypoxia-modifying treatment modalities [18]. A strategy that combines both AR and hypoxia modification is Accelerated Radiotherapy with CarbOgen (98% O_2; 2% CO_2) and Nicotinamide (ARCON) [19]. Results from clinical trials with ARCON show high locoregional control rates, in particular for oropharynx and larynx tumours [20,21].

Both irradiation [22] and hypoxia [13] can enhance phosphorylation of EGFR thereby regulating intrinsic DNA-repair mechanisms [10] and cellular proliferation. We therefore hypothesized that EGFR could modulate the tumour response to accelerated radiotherapy with hypoxia modification. The purpose of our study is to investigate the predictive value of EGFR expression for ARCON in patients with advanced laryngeal carcinoma using material from a recently completed trial randomizing between AR and ARCON [21].

Materials and Methods

Patients and treatment

Three-hundred-and-forty-five patients with advanced laryngeal carcinoma were included in a randomized trial comparing AR and ARCON between April 2001 and February 2008 at seven centres for head and neck oncology (six from the Netherlands and one from the UK). The eligibility criteria were published previously [21]. Approval from the local Ethics Committee of the Radboud University Nijmegen Medical Centre was obtained and all patients gave written informed consent. Pre-treatment paraffin-embedded biopsies were retrieved for immunohistochemical staining.

Immunohistochemistry

Sections from tumour biopsies were stained for EGFR expression as described previously with minor modifications [23]. Briefly, sections of 5μm were cut, deparaffinised and rehydrated through a graded ethanol series. Sections were incubated with proteinase-K (DAKO, Glostrup, Denmark) at 37°C. The primary antibody used was mouse anti-EGFR (DAKO M7239, Glostrup, Denmark) diluted 1:50 in PAD. The secondary antibody was a biotinylated F(ab)'2-donkey anti-mouse IgG (Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA), diluted 1:200 in PBS. Sections were counterstained with haematoxylin.

Image acquisition and quantitative analysis

All patient tissue sections were scanned with a Leica DM 6000 microscop (monochrome CCD camera (Retiga SRV) with an RGB filter (Slider module, QImaging, Burnaby, BC, Canada)) using IPlab imaging software (Scananalytics Inc., Fairfax, VA, USA). EGFR signal was scanned at 100X magnification. For every scanning session a background image was recorded. Extraction and separation of the individual colours from the DAB (brown) and haematoxylin (blue) signals was conducted using the RGB linear unmixing module in the TRI2-software (Randall Division and Gray Cancer Institute, London, UK). Based on a haematoxylin/eosin stained consecutive section, the tumour area of each section was delineated (necrotic areas and artefacts excluded). The EGFR fraction was defined as the tumour area positive for EGFR relative to the total tumour area. EGFR assays were performed blinded to the clinical endpoint.

Statistical analysis

Statistical analyses were performed on a Macintosh computer using Prism 4.0c (Hearne Scientific Software, Dublin, Ireland) software package. Comparison of baseline characteristics between patient groups was performed using the χ^2-test for categorical variables, and using the independent Students *t*-test for continuous variables. Correlations between the EGFR fraction and ordinal tumour characteristics

(T-classification, N-classification, and histopathological grade) were assessed using the Spearman and the Kruskal-Wallis tests. To determine correlations between EGFR expression level and survival (locoregional control (LCR) and disease-specific survival (DSS)), Kaplan-Meier graphs with log-rank testing were used after dichotomizing the patients into two groups based on low and high EGFR fractions. $p \leq 0.05$ was considered indicative of statistical significance. Multivariate analysis was performed using Cox proportional hazards analysis. Data are presented as hazard ratios (HR) with 95% confidence intervals (CI).

Results

Patients and treatment

A total of 345 patients were randomized. From these, 73 patients were excluded from the current analysis, 39 because biopsy material could not be retrieved, 24 biopsies contained no or very little invasive carcinoma, and 10 because of poor quality due to mechanical damage during biopsy procedure or poor staining quality. Thus, 272 histological confirmed squamous cell carcinomas of the larynx were available for analysis. The minimal duration of follow-up for all patients was two years with a median of 52 months for surviving patients. Patient and tumour characteristics were not significantly different between the AR and ARCON groups (Table 1). Treatment schedules and patient outcome have been published previously [21].

EGFR expression and correlation with tumour characteristics

Most tumours showed membranous EGFR expression (median fraction 0.4), but there was a wide range (0.00-0.93) (Figures 1 and 2). Based on the bimodal distribution, patients were dichotomized at a fraction of 0.43, resulting in a low EGFR fraction group (n=144, 53%) and a high EGFR fraction group (n=128, 47%). There was no correlation between EGFR levels and clinical parameters except for a difference between EGFR high and low groups with regard to N-classification. Tumours with high EGFR expression were more frequently lymph node positive at time of presentation (Table 1).

Table 1. Patient and tumour characteristics of 272 laryngeal tumour patients

	All (%)	Treatment (%)			EGFR expression		
		ARCON (n=138)	AR (n=134)	*p*	EGFR low	EGFR high	*p*
Age							
Median (range)	60.6 (38 – 88)	60.8 (42 – 84)	60.1 (38– 88)	0.65	60.8 (39-83)	62.8 (38-88)	0.09
Gender							
male	219 (81)	116 (84)	103 (77)	0.13	112 (78)	107 (84)	0.23
female	53 (19)	22 (16)	31 (23)		32 (22)	21 (16)	
Primary site							
glottic	115 (42)	60 (43)	55 (41)	0.68	58 (40)	57 (45)	0.48
supraglottic	157 (58)	78 (57)	79 (59)		86 (60)	71 (55)	
T-classification							
T2	99 (36)	46 (33)	53 (39)		50 (35)	49 (38)	
T3	133 (49)	73 (53)	60 (45)	0.40	76 (53)	57 (45)	0.33
T4	40 (15)	19 (14)	21 (16)		18 (12)	22 (17)	
N-classification							
N0	178 (65)	91 (66)	87 (65)	0.79	104 (73)	74 (58)	**0.01**
N+	94 (35)	47 (34)	47 (35)		40 (27)	54 (42)	
Histopathological differentiation grade							
well	17 (6)	9 (7)	8 (6)		8 (5)	9 (7)	
moderate	153 (56)	78 (56)	75 (56)		79 (55)	74 (58)	
poor	47 (17)	29 (21)	18 (13)	0.18	26 (18)	21 (16)	0.71
n.k.	55 (20)	22 (16)	33 (24)		31 (22)	24 (19)	

n.k. not known

Table 2. 5-years loco-regional control and disease-specific survival (%) and hazard ratio of patients treated with ARCON versus AR by EGFR expression fraction.

Outcome end points		AR	ARCON	*p*	HR (95% CI)
Disease-specific survival	Low EGFR	82	91	0.08	0.38 (0.12-1.12)
	High EGFR	77	78	0.54	1.23 (0.58-2.86)
Loco-regional control	Low EGFR	69	88	**0.009**	0.34 (0.14–0.79)
	High EGFR	75	71	0.85	1.07 (0.53-2.15)

HR, hazard ratio; CI, confidence interval

Table 3. Multivariate analysis of clinical parameters and EGFR fraction

Variables	DSS AR		DSS ARCON		LRC AR		LRC ARCON	
	p	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)
T-classification (T_2 vs T_3 vs T_4)	0.156	1.54 (0.85-2.79)	0.726	1.14 (0.55-2.39)	0.349	0.79 (0.49-1.29)	0.261	1.39 (0.78-2.51)
N-classification (N_0 vs N_+)	**0.019**	1.33 (1.05-1.69)	**0.010**	1.43 (1.09-1.87)	0.198	1.15 (0.93-1.41)	0.742	0.95 (0.69-1.25)
Histopathological grade (poor vs moderate vs well)	0.571	0.96 (0.81-1.12)	0.367	0.88 (0.66-1.17)	0.321	0.94 (0.84-1.06)	0.283	1.08 (0.34-1.25)
EGFR fraction (low vs high)	0.912	0.95 (0.40-2.26)	**0.05**	3.00 (0.99-9.16)	0.507	0.79 (0.39-1.58)	**0.048**	2.43 (1.01-5.87)

HR, hazard ratio; CI, confidence interval

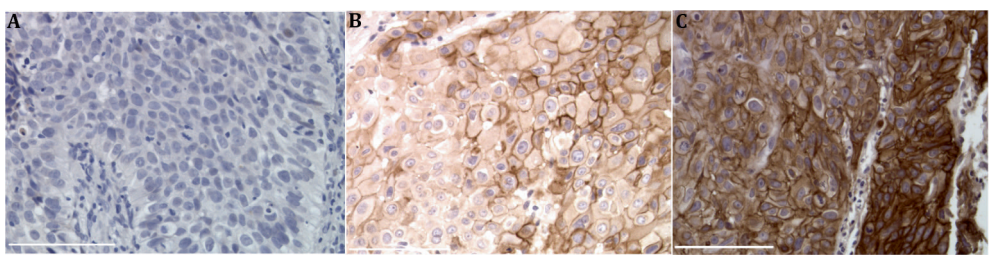

Figure 1. Range of the EGFR expression in laryngeal carcinoma (A) no expression; (B) intermediate expression and (C) high expression at 400x magnification. Scalebars represents 100 μm.

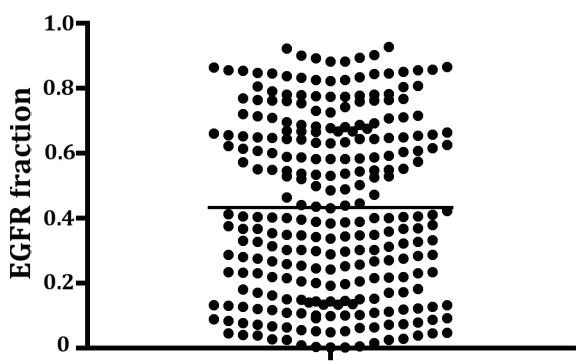

Figure 2. Distribution of EGFR expression based on EGFR fraction of whole tissue section in 272 laryngeal tumours analysed in the ARCON trial. Line represents cut-off.

Associations between EGFR expression and treatment outcome

No difference in 5-year disease specific survival (DSS) or locoregional control (LRC) was found between low and high EGFR expressing tumours in the AR arm (DSS 81% versus 78% and LRC 69% versus 75% respectively) (Figure 3a, c). In the ARCON arm, however, EGFR fraction was a significant prognostic factor for DSS and LRC. Patients with low EGFR levels had a better 5-year DSS (92% versus 77% p=0.01) and LRC (88% versus 72% p=0.02) compared to patients with high EGFR (Figure 3b, d).

Table 2 shows the same data, now comparing AR against ARCON in the subgroups of patients with low and high EGFR fractions. ARCON improves 5-year LRC relative to AR in patients with low EGFR fraction (88% for ARCON versus 69% for AR p=0.009; Hazard Ratio (HR) 0.34) but there was no effect of ARCON in patients with high EGFR fraction. The same phenomenon was observed for DSS, albeit that the difference was borderline significant (p=0.08).

The multivariate analysis (Table 3) confirmed EGFR fraction as an independent predictive factor for DSS and LRC in the patients treated with ARCON. After correction for T-, N-classification and histopathological grade, EGFR expression remained associated with LRC and DSS in the ARCON treatment arm (HR 2.43 CI 1.01-5.87 p=0.048 and HR 3.00 CI 0.99-9.16 p=0.05 respectively).

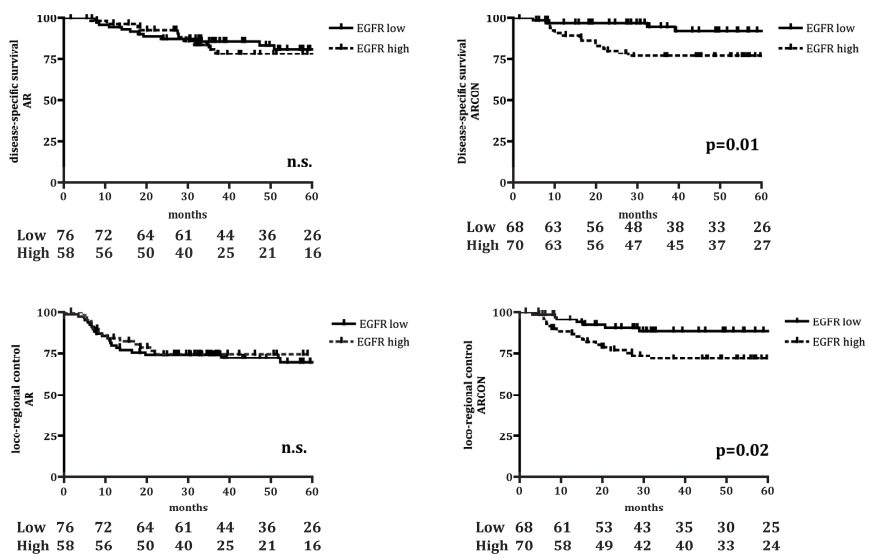

Figure 3. Kaplan-Meier estimates of (A) disease-specific survival in AR arm versus (B) in ARCON arm and (C) locoregional control in AR arm versus (D) in ARCON arm. Stratification is by the cut-off value of EGFR fraction. Comparison by log-rank test.

Discussion

ARCON, a new treatment option for larynx carcinoma, combines AR with hypoxic modifiers to counteract enhanced tumour cell repopulation and reduce intratumoural hypoxia [19]. Selection of patients for new treatments is traditionally based on standard clinical and histopathological tumour characteristics. Predictive assays, based on molecular tumour markers and the oxygenation status of tumour cells, may provide better tools to select patients for new treatment approaches. EGFR is involved in all mechanisms of radioresistance and we therefore hypothesized that EGFR could be a powerful biomarker to predict tumour response to biology-based radiotherapy modifications. In the current study, we demonstrated that tumours with low EGFR expression respond better to ARCON compared to AR. EGFR expressing tumours were more likely to be node positive. However, as ARCON is most effective for regional control [21], this putative bias would indicate that the actual effect may be even more pronounced. In tumours with a high EGFR fraction, adding hypoxia modification to AR had no additive beneficial effect on outcome.

Biopsies of patients with advanced laryngeal carcinoma were examined for EGFR expression. Similarly to other studies [4,24-26], we observed a wide variation in the EGFR expression levels. Most of the previous studies found a correlation between high (above median) EGFR expression and worse clinical outcome. One of the larger series including 155 head and neck cancer patients demonstrated that EGFR expression was a strong and independent prognostic indicator for LRC (HR 1.95 p=0.002) and overall survival (HR 1.75 p=0.006) [4]. In this study, all patients were treated with conventionally fractionated radiotherapy [4]. Two subsequent randomized trials consistently demonstrated that differences in outcome between high and low EGFR expressing tumours disappeared when accelerated radiotherapy was employed [26,27]. The latter improves tumour control only in tumours with high EGFR-expression levels [26-28], most likely through suppression of EGFR-induced tumour cell proliferation.

In the current study, both for the patient's cohorts with high as well as low EGFR expressing tumours, 5-year LRC in the AR arm was around 70%, confirming the observations in the previously mentioned studies that acceleration radiation can counteract EGFR-associated radiation resistance [26-28]. Also in the tumours with high EGFR-expression treated with ARCON, a LRC rate of about 70% was obtained. However, the LRC for tumours with low EGFR levels treated with ARCON was significantly improved (88%, p=0.02). So, patients with low EGFR expressing tumours benefit from ARCON, whereas patients with high EGFR expressing tumours do not. Multivariate analysis indicated that EGFR expression is an independent predictor of LRC and DSS in patients treated with ARCON.

Table 4. Chi-square

	EGFR low	EGFR high
Pimo low	21	28
Pimo high	8	14

p=0.402

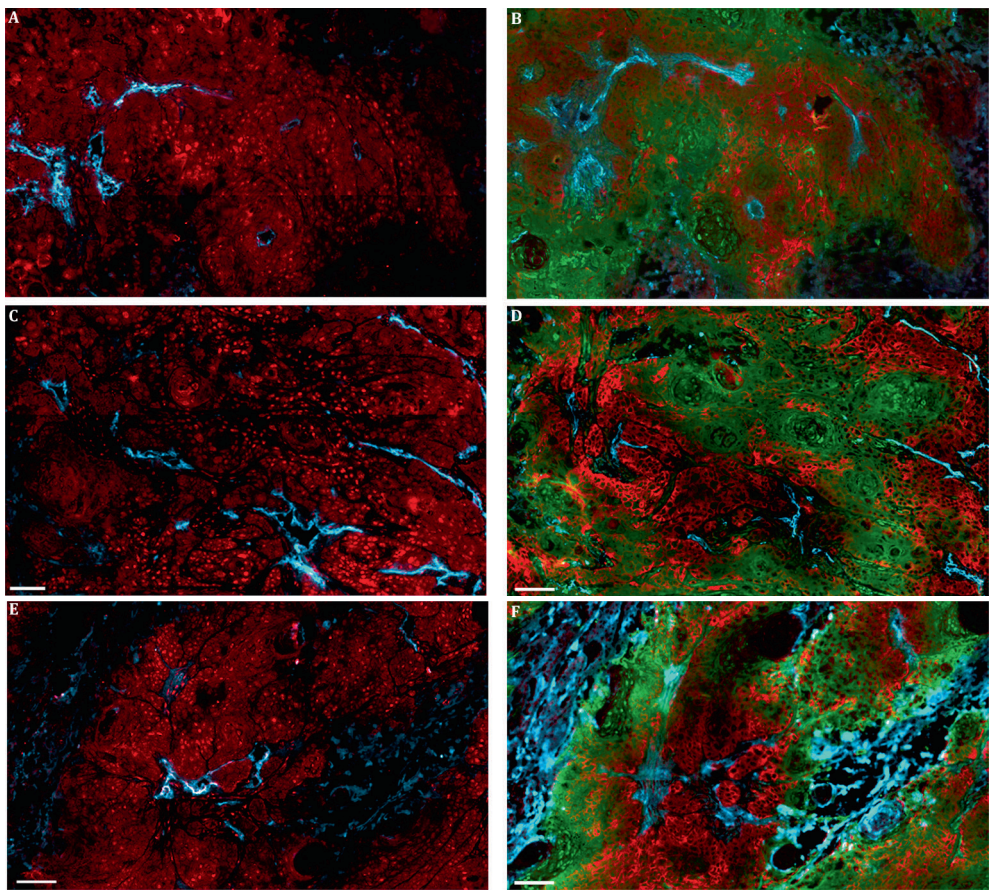

Figure 4. Fluorescence image from three xenografted larynx carcinomas showing (A, C & E) HIF-1α (red) with vessels (blue) and (B, D & F) an adjacent section pimonidazole (green) in combination with EGFR (red) and vessels (blue). HIF-1α expression is present in pimonidazole positive (hypoxic) and negative (normoxic) areas. 100x magnification. Bar represents 100 μm.

From studies performed with EGFR inhibitors it is known that EGFR signalling is associated with the hypoxia response. Blocking EGFR can reduce intratumoural hypoxia possibly by normalization of the irregular dysfunctional tumour vasculature, thereby improving perfusion and oxygen delivery [29-31]. A major pathway in the hypoxia response is the HIF-pathway. HIF-1 is a heterodimer consisting of two subunits: HIF-1α and HIF-1β. Under normoxic conditions, HIF-1α is rapidly degraded, but under hypoxia it is stabilized [32]. Interestingly, EGFR itself can induce the HIF-1 pathway in an oxygen-independent way by stabilizing HIF-1α, thereby activating the same target genes [33]. Tumour cells with high pre-treatment EGFR expression levels might be better able to rapidly activate downstream survival pathways, resulting in activation of the HIF-pathway, thereby thwarting the hypoxia modifying effect of ARCON. Low EGFR expressing tumours possibly lack this capacity and thus might be more likely to respond to ARCON. ARCON might reduce activation of the HIF-1 pathway in hypoxic tumours but possibly cannot counteract the EGFR-induced HIF-response in normoxic tumour cells. Therefore, in high EGFR expressing tumours, the defence mechanism through the HIF-1 pathway can still be active despite hypoxia modification. Figure 4 illustrates that in laryngeal tumours there is expression of HIF-1α present in normoxic and EGFR-positive tumour areas, supporting this EGFR-dependent HIF-response. Due to technical limitations HIF-1α could only be assessed qualitatively and not quantitatively. No correlation was found between overall fractions of EGFR and pimonidazole (Table 4). In the absence of hypoxia upregulation of HIF-1 may drive cells toward glycolysis, known as the Warburg effect, providing a growth advantage for tumour cells and resistance to ionizing radiation [11]. Hypoxia-independent but EGFR-dependent upregulation of HIF-1α could account for the resistance of EGFR expressing tumours to hypoxia-modifying treatment. This hypothesis will be further explored in animal experiments. EGFR inhibitors also have an effect on the reoxygenation of tumour cells [34,35]. Possibly, in tumours with high EGFR expression signalling must be inhibited prior to ARCON treatment in order to benefit.

Conclusion

Current knowledge provides strong evidence that EGFR signalling plays an important role in the regulation of tumour cell survival during and after radiation treatment. EGFR evolved from a prognostic marker for patient outcome after conventional fractionated radiotherapy to a predictive biomarker for the effect of accelerated radiotherapy. In the current study we demonstrate that EGFR expression is also predictive for the response to hypoxic modification but with a reverse association. In patients with laryngeal carcinomas with low EGFR levels outcome can be further improved with ARCON, while there is no advantage of ARCON for patients with high

EGFR expressing tumours. In the latter, the effect of EGFR inhibition in combination with hypoxia modification needs to be explored.

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Chapter 4

Spatial relationship of phosphorylated epidermal growth factor receptor and activated AKT in head and neck squamous cell carcinoma

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Abstract

Overexpression of EGFR correlates with decreased survival after radiotherapy in head and neck squamous cell carcinoma (HNSCC). However, the contribution of the activated form, pEGFR, and its downstream signalling (PI3-K/AKT) pathway is not clear yet.

Fifty-eight patients with HNSCC were included in the study. pEGFR, pAKT, hypoxia, and vessels were visualized using immunohistochemistry. Fractions (defined as the tumour area positive for the respective markers relative to the total tumour area) were calculated by automated image analysis and related to clinical outcome.

Both pEGFR (median 0.6%, range 0-34%) and pAKT (median 1.8%, range 0-16%) expression differed between tumours. Also, a large variation in hypoxia was found (median pimonidazole fraction 3.9%, range 0-20%). A significant correlation between pEGFR and pAKT (r_s 0.44, p=0.004) was seen, however, analysis revealed that this was not always based on spatial coexpression. Low pAKT expression was associated with increased risk of regional recurrence (p<0.05, log-rank) and distant metastasis (p=0.04).

The correlation between expression of pEGFR and pAKT is, indicative of activation of the PI3-K/AKT pathway through phosphorylation of EGFR. Since not all tumours show coexpression to the same extent, other factors must be involved in the activation of this pathway as well.

Introduction

The Epidermal Growth Factor Receptor (EGFR) is a transmembrane tyrosine kinase that can be activated in response to binding of ligands or irradiation [1]. Phosphorylated EGFR (pEGFR) results in activation of various downstream pathways e.g. the phosphatidyl-inositol-3' kinase (PI3-K)/Akt pathway [2]. After phosphorylation, AKT (pAKT) translocates to the cytoplasm and nucleus leading to transcription of genes responsible for cell cycle progression, cellular proliferation, DNA-damage repair, and apoptosis, processes contributing to tumour progression [3-9]. Genes involved in the cellular response to hypoxia such as hypoxia-inducible factor-1α (HIF-1α) are also activated by pAKT [10].

Several investigators have explored the role of EGFR or (p)AKT expression in patients with HNSCC. Mostly, they found a strong correlation between high EGFR expression and poor clinical outcome [11-15]. In addition, pAKT was found to be a significant predictor for local control [16,17]. There are indications that activation of the EGFR-PI3-K/AKT pathway plays a role in radiation resistance with subsequently poor treatment outcome [18-21]. EGFR as well as pAKT are highly expressed in the majority of patients with head and neck squamous cell carcinoma (HNSCC) [6,15]. Overexpression of EGFR does not necessarily implicate phosphorylation of the receptor and activation of downstream pathways and there are, to our knowledge, no clinical studies relating pEGFR expression to clinical outcome. Besides activation through EGFR, the PI3-K/AKT pathway can be activated by several other mechanisms. These include activation through other receptor tyrosine kinases (RTKs), mutations in oncogenes upstream such as *ras*, loss of Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [22], or amplifications or mutations of the gene PIK3CA, encoding the catalytic subunit of PI3K, or AKT itself [23,24].

The purpose of this study was to examine the relative contribution of activated EGFR on the activation of the PI3-K/AKT pathway by investigating expression levels of pEGFR and pAKT, and spatial coexpression of these two markers. Hypoxic tumour cells with an activated EGFR-PI3-K/AKT pathway could have a survival advantage after treatment with radiotherapy. Therefore, the relationship between pEGFR, pAKT, and hypoxia was also investigated in these tumours.

Patients and Methods

Patients

Between May 1998 and November 2001, 58 patients with HNSCC were included in our study at the Radboud University Nijmegen Medical Centre, Nijmegen. Patients with primary stage II to IV squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx were included. Patients were treated with radiotherapy alone or in combination with other treatment modalities such as chemotherapy or surgery. A written informed consent and approval from the local ethics committee was

obtained. Approximately 2 h before taking a biopsy, patients received a 20 min intravenous (i.v.) infusion of the hypoxia marker Hypoxyprobe-1 (500 mg/m^2) (pimonidazole hydrochloride; NPI Inc., USA). A maximum dose of 1 g was given to patients >2m^2. Biopsies were taken for routine diagnostic purposes before treatment and additional biopsies were taken for multiple marker analyses. The latter were immediately snap frozen in liquid nitrogen until immunohistochemical processing.

Immunohistochemistry

From the biopsy material, sections of 5 μm were cut, mounted on poly-l-lysine coated slides and stored at -80 °C. Prior to staining the sections were fixed in acetone of 4 °C for 10 min and rehydrated in phosphate buffered saline (PBS Klinipath, The Netherlands). Afterwards sections were incubated in primary antibody dilution (PAD, GeneTex Inc., USA) for 5 min at room temperature. Between all consecutive steps of the staining procedure, sections were rinsed in PBS three times for 5 min. The sections were incubated overnight at 4 °C with rabbit anti-pAKT antibody (Ser473) and goat anti-pEGFR antibody (Santa Cruz Biotechnology Inc., USA) diluted 1:50 and 1:100 in PAD respectively. Adjacent sections were incubated overnight at 4 °C with rabbit anti-pimonidazole antibody (J.A. Raleigh, Department of Radiation Oncology and Toxicology, University of North Carolina, USA) and goat anti-pEGFR antibody 1:1000 and 1:100 in PAD respectively. The second incubation was for 30 min at 37 °C with donkey anti-rabbit Alexa488 (Molecular Probes, The Netherlands) and donkey anti-goat Cy3 (Jackson Immunoresearch Laboratories Inc., USA) diluted 1:600 in PBS. The sections were stained for vessels by incubation with the mouse antibody PAL-E (Euro Diagnostica, The Netherlands) diluted 1:10 in PAD followed by incubation for 30 min at 37 °C with chicken anti-mouse Alexa647 antibody (Molecular Probes) diluted 1:100 in PBS. The monoclonal antibody PAL-E is a marker for human endothelium, especially useful in frozen tissue sections. After the staining procedure, the sections were mounted in fluorostab (ProGen Biotechnik GmbH, Germany).

Image acquisition

The tissue sections were scanned with a digital image processing system consisting of a high-resolution 12-bit CCD camera (Micromax, Roper Scientific Inc., USA) on a fluorescence microscope (Axioskop, Zeiss, Germany) and a computer-controlled motorized stepping stage. Image processing was done using IPLab software (Scanalytics Inc., USA) on a Macintosh computer, as described earlier [25]. Each tissue section was sequentially scanned for the pimonidazole, pEGFR, pAKT and vessel signals at 200x magnification. The resulting composite gray value images were converted to binary images for further analysis. Thresholds for the fluorescence signals were interactively set at intensities where the steepest gradient occurred between background and foreground intensity levels. The corresponding composite

binary images were superimposed into one pseudocoloured image for visual evaluation.

Analysis

With H&E staining of a consecutive section, the tumour area of each section was delineated. This area was used as a mask in further analysis from which non-tumour tissue, necrotic areas, and artifacts were excluded. To calculate the amount of coexpression of pEGFR and pAKT, a binary closing operation on the pEGFR signal was done. The fractions of pEGFR (FpEGFR), pAKT (FpAKT) and hypoxia (HFpimo) were defined as the tumour area positive for the markers divided by the total tumour area. The vascular density (VD) was calculated as the number of vascular structures per square millimeter. The fraction of pAKT expressing cells positive for pEGFR was defined as the area that stained positive for both pEGFR and pAKT divided by the total pAKT-positive area ($FpAKT_{[pEGFR]}$).

To quantify the distribution of hypoxia, pEGFR, and pAKT in relation to the vasculature, zones were chosen at increasing distance from the surface of the nearest vessel (0-50 μm, 51-100 μm, 101-150 μm, 151-200 μm, and 201-250 μm). Hypoxic fraction, as well as fractions pEGFR and pAKT, and $FpAKT_{[EGFR]}$ were calculated within these vasculature zones.

Statistics

Statistical analyses were done on a Macintosh computer using Prism 4.0c (Hearne Scientific software, Ireland) software package. Data were log transformed passing normality testing. Correlations between parameters were assessed using the Pearson correlation test. To determine correlations between these parameters and categorical tumour characteristics (site, T-classification, N-classification, and histopathological grade) the Spearman correlation and the Kruskal-Wallis tests were used. To determine correlation between the parameters and risk (local and regional control, metastasis-formation) Kaplan-Meier graphs with log-rank testing was used. $p \leq 0.05$ was considered indicative of statistical significance.

Results

Patients and treatment

A total of 58 patients were included in this study. Pimonidazole was given to all patients before biopsy and none of them had adverse reactions. Table 1 shows the clinical characteristics of the patients. Seventeen biopsies were excluded from the analysis, six because they contained no or very little invasive carcinoma, eight because of poor quality due to mechanical damage during biopsy procedure or poor staining quality and three because the histological diagnosis was not squamous cell carcinoma. Thus, 41 histological confirmed squamous cell carcinomas were used for analysis.

There was no significant difference between the clinical parameters of the included and excluded patients. The median duration of follow-up for all patients was 25.7 months and for surviving patients 86.6 months.

Table 1. Patient and tumour characteristics of 41 HNSSC

Age	
Mean (range)	58 (36-85)
	Number (%)
Gender	
Male	35 (85)
Female	6 (15)
T-classification	
T2	14 (34)
T3	17 (41)
T4	10 (24)
N-classification	
N0	11 (27)
N+	30 (73)
Tumour site	
Larynx	20 (49)
Hypopharynx	10 (24)
Oropharynx	9 (22)
Oral cavity	2 (5)
Differentiation grade	
Good	2 (5)
Moderate	21 (51)
Poor	18 (44)

Immunohistochemistry pEGFR, pAKT, pimonidazole and vessels

Every biopsy was stained for pEGFR, pAKT or pimonidazole, and vessels. All markers gave bright fluorescent staining with little background except in areas of necrosis and stromal components of the tumour. Pimonidazole binding and pAKT expression was observed in the cytoplasm, while pEGFR staining was limited to the cell membrane (Figure 1). Coexpression of pEGFR and pAKT ($FpAKT_{[pEGFR]}$) could identify tumour cells in which activated EGFR is linked to the activation of the PI3-K/AKT pathway. Figure 2 illustrates tumour cells in which pEGFR is coexpressed with pAKT, but also tumour cells with mismatch all within the same tumour section. The median values and range for all quantitatively measured parameters are summarized in table 2. A moderate but significant correlation between the overall expression of pEGFR and pAKT in the different tumours was found (r_s=0.4 p=0.004) (Figure 3). There was no correlation between pimonidazole binding and expression of pEGFR or pAKT. As expected, pimonidazole binding increased with increasing distance from the blood vessels with highest fractions at >200 μm from the nearest vessel (Figure 3B). Expression of pEGFR and pAKT was found in normoxic as well as hypoxic tumour

cells. Most $FpAKT_{[pEGFR]}$ was observed at close distance to the vessels, mainly below 100 μm, while less overlap was seen in the more hypoxic areas at greater distance (Figure 3B).

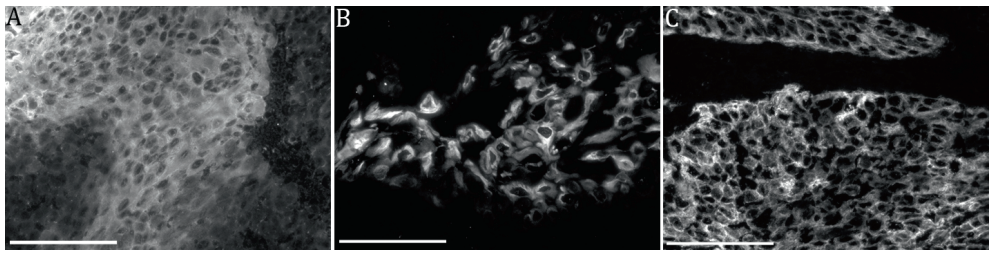

Figure 1. Fluorescence image (grey values) at 400x magnification of (A) cytoplasmatic pimonidazole binding, and (B) pAKT expression, and (C) membranous pEGFR expression. Bar represents 50 μm.

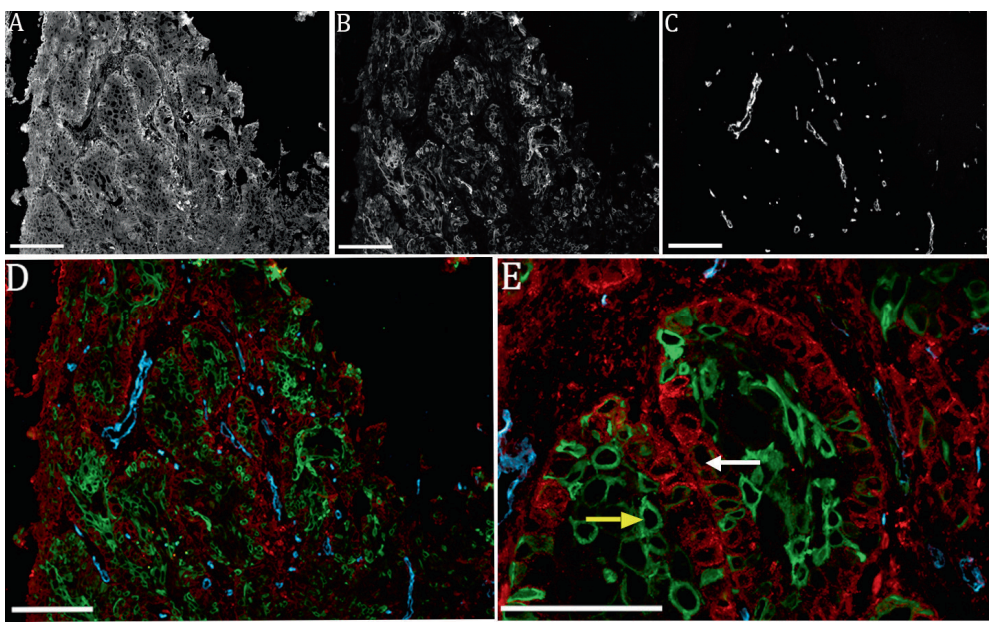

Figure 2. Fluorescence image showing membranous pEGFR and cytoplasmatic pAKT staining of a squamous cell carcinoma of the larynx. Grey scale image of (A) pEGFR, (B) pAKT, and (C) vessels (100x magnification). (D) Composite fluorescence image at 100x magnification and (E) detailed image at 400x magnification. Red: pEGFR, green: pAKT, blue: vessels. White arrow: coexpression, yellow arrow: cell with mismatch. Bar represents 100 μm (A-D) and 50 μm (E).

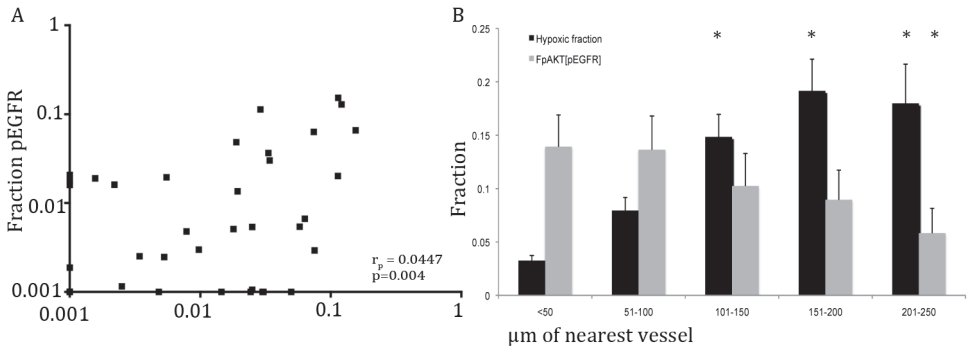

Figure 3. Fraction pEGFR versus pAKT in 41 head and neck squamous cell carcinomas (A) and distribution of HF_{pimo} (black bars) and $FpAKT_{[pEGFR]}$ (grey bars) as a function of distance (µm) of nearest vessel (B). Plots show average values of 41 tumours (+/- SEM). *Significant difference compared to <50 µm.

Table 2. Overall values for all parameters in 41 patients with HNSSC, measured with image analysis

	HFpimo (%)	FpEGFR (%)	FpAKT (%)	$FpAKT_{[pEGFR]}$	VD (N/mm²)
Median	3.9	0.6	1.5	1.8	293
Range	0-20	0-34	0-16	0-78	80-1440
No. positive biopsies	31 (76%)	27 (66%)	30 (74%)	-	41 (100%)

Correlation between molecular markers and tumour characteristics

A significant correlation was found between pAKT expression and N classification (p=0.001). Figure 4 shows that tumours with lymph node metastases had lower pAKT expression compared to lymph node negative tumours (median FpAKT 0.5% versus 5.8%).

Apart from a moderate, positive correlation of $FpAKT_{[pEGFR]}$ with T-classification (data not shown), no other correlations between the individual markers or coexpression of the markers and T- and N-classification, tumour site, or histological grade were found.

Correlation between microenvironmental parameters and outcome

Patients were dichotomized based on median expression values. A low pAKT expression was associated with a significantly shorter time to regional recurrence and on metastasis formation (log-rank p=0.04 resp. p<0.05) (Figure 5). The 5-year

regional recurrence and metastasis-risk was 40% and 64% for patients with low pAKT expression versus 6% and 36% for patients with high pAKT expression. There was no association between pEGFR expression and regional control or distant metastasis. Local control was not associated with pEGFR or pAKT expression.

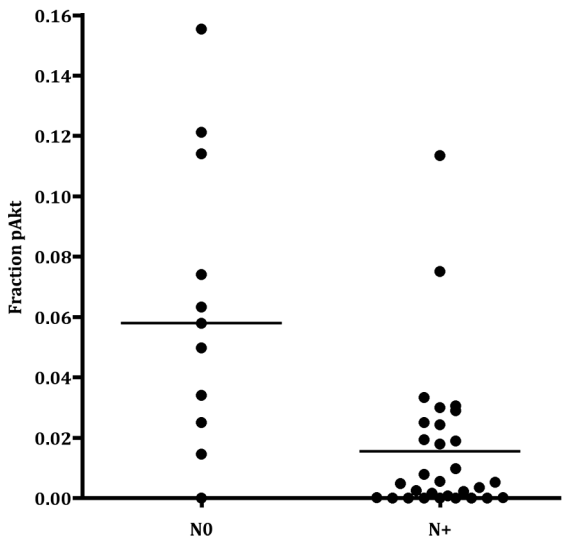

Figure 4. Expression of pAKT in node positive (N+) versus node negative (N0) tumours. Lines represent the median; $p = 0.001$.

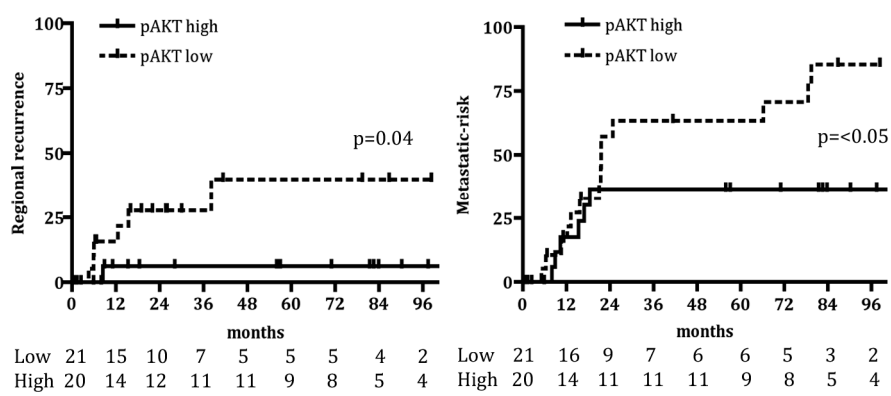

Figure 5. Kaplan-Meier estimates of regional recurrence (A) and metastasis-risk (B). Stratification by median pAKT value.

Discussion

Overexpression of EGFR is commonly found in HNSCC [26]. Previous studies have revealed that higher expression of EGFR as well as pAKT is associated with a poor response to radiotherapy [13,16,17,20,21,27]. Activation of EGFR may result in activation of the PI3-K/AKT pathway. However, it is unknown to which extent this pathway is involved in radiation resistance in vivo. In our study, a heterogeneous group of head and neck cancer patients (with regard to site, classification, and treatment) was investigated for expression of pEGFR and pAKT, in relation to the microenvironment. In general, total EGFR is highly expressed (>90%) in HNSCC [11]. In our study the activated form, pEGFR, was observed in 66% of the tumours and expression of pAKT was found in 74% of the patients. The large variation in expression levels is in agreement with previous studies, which showed a high variation in expression of pEGFR [28-30] and pAKT [16,31,32] in other solid tumours as well. In addition, in the current study a positive relationship was found between pEGFR and pAKT, suggesting that EGFR signalling is one of the upstream regulators of the PI3-K/AKT pathway in HNSCC. A correlation between pEGFR and pAKT was found previously in patients with nasopharyngeal carcinoma [30]. Of note, in that study a clear distinction was made between the two known phosphorylation sites of AKT (Thr308 and Ser473), while in the present study one phosphorylation site of pAKT (Ser473) was investigated, in consensus with most other studies. The authors suggested that the increased activation of PI3-K/AKT signalling in nasopharyngeal cancer is most likely due to overexpression of pEGFR rather than mutations of the PIK3CA gene since they detected no mutations.

Coexpression of pEGFR and pAKT might identify tumour cells in which EGFR is responsible for the activation of AKT and may therefore be a predictive factor for the response to EGFR-inhibitors, radiotherapy, or both. The PI3-K/AKT pathway triggers a cascade of responses, which are involved in all major radiation resistance mechanisms [18]. In the present study, a large variation was found in coexpression of pEGFR and pAKT, indicating that in head and neck cancer not all tumour cells that express pAKT are activated by EGFR and vice versa. Activation of AKT can occur by several mechanisms, independent of EGFR activation. These include amplifications or mutations of the gene PIK3CA [23,24], amplifications of AKT, activation by other RTKs or hetrodimerization of other ErbB family members [33], and decreased expression of PTEN [22]. PTEN, which acts a tumour suppressor, limits the activity of PI3K pathway and loss of PTEN results in unrestrained activation of AKT [5] and upregulation of the downstream proteins responsible for all major cancer growth mechanisms, thereby increasing radiation resistance. On the other hand, our results, i.e. pEGFR expression without pAKT expression, indicate that not in all cells activation of EGFR necessarily leads to activation of the downstream PI3-K/AKT pathway. Apart from of the PI3-

K/AKT pathway, EGFR can activate other cell survival pathways including mTOR, RAS/MAPK, and the STAT/JAK pathway [34].

Coexpression, suggesting activation of AKT through pEGFR, is highest in parts of the tumour at a relatively short distance (below 100 μm) from the vessels. Figure 3B shows that in severely hypoxic tumour cells, pEGFR is of little influence on the activation of the PI3-K/AKT pathway. This suggests that activation of AKT in hypoxic cells is more commonly due to the other mechanisms previously mentioned. Also, activation of EGFR without pAKT expression was present, possibly leading to EGFR-dependent activation of other pathways. It has been described earlier that hypoxia induces expression of EGFR, and in turn EGFR might enhance the cellular response to hypoxia and may therefore act as survival factor for hypoxic cells [35,36]. In response to hypoxia, cells have the ability to undergo adaptive changes [37], which can result in survival of the tumour cells and activation of the EGFR-PI3-K/AKT pathway under reoxygenated conditions. Even a small proportion of these reoxygenated cells may repopulate the tumour after treatment with radiotherapy. Recent studies show that inhibition of EGFR by the monoclonal antibody C225 leads to a decreased AKT phosphorylation, reduces tumour repopulation during radiotherapy and contributes to an improvement of tumour control [38,39].

An inverse correlation between pAKT and N-classification was found. Patients with negative lymph nodes have a significantly higher expression of pAKT compared to patients with positive lymph nodes. This was previously also reported for patients with gastric carcinomas [40]. In addition to these findings, we found that low pAKT expression is associated with worse regional control, which, obviously, is directly linked to the higher incidence of lymph node metastases at presentation, and metastasis formation. These results are in contrast with several clinical studies were high pAKT expression is a prognostic factor for poor disease control [17,20,21]. However, recent studies demonstrated a role of pAKT in invasion and metastasis. One study showed that activation of AKT1 can suppress tumour invasion and lung metastasis formation in a mammary mice model [41]. Their hypothesis is that AKT1 may hinder metastasis by preventing the degradation of the extracellular matrix and promoting differentiation of the mammary epithelium. In another study, with a breast epithelial cell line, downregulation of AKT1 enhanced EGF stimulated migration of cells [42]. The enhanced migration was accompanied by changes in protein expression that are consistent with epithelial-mesenchymal transition (EMT) characterized by loss of cell adhesion. Whether pAKT has the same role in HNSCC is under investigation.

In conclusion, in a group of 41 head and neck carcinomas the presence of pEGFR, pAKT expression in relation to hypoxia and blood vessels was determined. Our data suggest that activation of the PI3-K/AKT pathway is only partly due to activation of EGFR in HNSCC. The high percentage of pEGFR and pAKT positive patients seen in this

study supports an important role for the EGFR-PI3-K/AKT pathway in the biology of head and neck cancer. Our data are consistent with a role for pAKT in cell migration and thwarting of metastasis. Currently, analysis of tumour material obtained from a randomized trial employing accelerated radiotherapy and oxygenation modification in patients with advanced laryngeal carcinoma (ARCON) [43] has started confirm these observations in a more homogenous group of patients.

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Chapter 5

Expression of E-cadherin and vimentin correlates with metastasis formation in head and neck squamous cell carcinoma patients

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Abstract

E-cadherin is a transmembrane glycoprotein, involved in cell-cell adhesion and epithelial-mesenchymal transition (EMT). Vimentin is highly expressed in mesenchymal cells and is positively correlated with increased metastasis. Here we set out to determine the expression of E-cadherin and vimentin in head and neck squamous cell carcinomas (HNSCC).

Twenty-six patients with primary stage II to IV HNSCC were included. E-cadherin and vimentin were visualized using immunohistochemistry, semi-automatically analyzed for expression patterns and correlated with the clinical behaviour of these tumours.

A large variation in E-cadherin and vimentin expression was observed between tumours (median 17% range 0-51% respectively median 0% range 0-20%). Tumours with low E-cadherin expression showed a significantly higher incidence of metastasis formation compared to tumours with high expression (81% versus 19%, p=0.004). Enhanced expression of vimentin was associated with a trend towards a higher metastatic risk (33% versus 77%) compared to tumours without expression of vimentin. All patients with low E-cadherin and high vimentin expression (an EMT-phenotype) developed distant metastases versus only 44% of the other patients (p=0.008).

Loss of E-cadherin and gain of vimentin may be associated with enhanced migration of tumour cells, leading to higher metastatic risk of HNSCC patients.

Introduction

Regional or distant metastasis formation is a major determinant in the prognosis of patients with head and neck squamous cell carcinoma (HNSCC). For these patients there is no curative treatment and they will die of their disease. Metastasis formation requires the spreading of cancer cells from their primary site to secondary locations in the body, the reattachment and growth at the new location. For tumour cells to migrate and form metastases, they must undergo changes in cell-cell adhesion, remodel cell-matrix adhesion sites and follow a chemoattractive path through the extracellular matrix; a phenomenon known as epithelial-mesenchymal transition (EMT) [1-3]. Loss of E-cadherin expression, leading to reduced cell-cell adhesion, as well as elevated levels of the mesenchymal marker vimentin, are distinctive events in EMT and common in metastatic carcinomas [1,2,4].

E-cadherin is a cell adhesion molecule present in the plasma membrane of most epithelial cells and has been implicated as a tumour suppressor in several types of human epithelial tumours, inhibiting migration and metastasis [3,5]. E-cadherin itself does not exhibit enzymatic activity. However, it has been shown that E-cadherin-mediated-cell-cell adhesion can trigger a ligand-independent activation of the EGFR, regulating important cell signalling pathways such as PI3-K/AKT and Extracellular Signal-Regulated Kinase (ERK) [6-8]. On the other hand, E-cadherin inhibits ligand-dependent activation of EGFR [9] (reviewed in Cavallaro 2011 [10]). Furthermore, increasing evidence indicates that the EGFR signalling pathways are able to regulate expression of the proteins involved in EMT [11,12]. Although some studies explored the expression of E-cadherin in HNSCC [13,14] and its relation with EGFR [15], it remains unclear whether there is a correlation between E-cadherin and the EGFR signalling pathways within tissue context.

Vimentin is a intermediate-sized filament that is highly expressed in mesenchymal cells and is commonly used to identify cancer cells undergoing EMT based on a positive correlation of vimentin expression with increased invasiveness and metastasis [4].

In HNSCC, radiotherapy is effective in early-stage tumours, but less effective for advanced tumours and only palliative in metastatic disease [16]. Loss of E-cadherin and gain of vimentin expression as well as activation of EGFR, are associated with tumour progression and EMT [4]. These considerations have led us to explore whether there is an association between E-cadherin and vimentin expression and the EGFR-PI3-K/AKT signalling pathway and/or metastasis formation in patients with head and neck cancer.

Patients and Methods

Patients

Twenty-eight patients with primary stage II to IV squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx or larynx, treated at the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands were included. Written informed consent was obtained from all patients after approval from the local ethics committee. Biopsies were taken for routine diagnostic purposes before treatment and during this procedure additional biopsies were taken from all patients for multiple marker analysis. The latter were immediately snap frozen in liquid nitrogen until immunohistochemical processing.

Immunohistochemistry

From the biopsy material, sections of 5 μm were cut, mounted on poly-l-lysine coated slides and stored at -80 °C. Prior to staining the sections were fixed in acetone of 4 °C for 10 min and rehydrated in phosphate buffered saline (PBS Klinipath, The Netherlands). Afterwards, sections were incubated in primary antibody diluent (PAD, GeneTex Inc., USA) for 5 min at room temperature. Between all consecutive steps of the staining procedure, sections were rinsed in PBS three times for 5 min. The sections were incubated overnight at 4 °C with goat anti-E-cadherin (Santa Cruz Biotechnology Inc., USA) 1:50 in PAD. The second incubation was for 30 min at 37 °C with donkey anti-goat Cy3 (Jackson Immunoresearch Laboratories Inc., USA) diluted 1:600 in PAD. The sections were stained for vessels by incubation with the mouse antibody PAL-E (Euro Diagnostica, The Netherlands) diluted 1:10 in PAD. Next, sections were incubated for 30 min at room temperature with rabbit anti-vimentin (Santa Cruz Biotechnology Inc.) 1:200 in PAD followed by incubation for 45 min at 37°C with donkey anti-rabbit Alexa488 (Molecular Probes, The Netherlands) and chicken anti-mouse Alexa647 antibody (Molecular Probes) diluted 1:100 in PAD. EGFR and pAKT (goat anti-EGFR and rabbit anti-pAKT 1:50, Santa Cruz Biotechnology Inc.) staining was combined with either E-cadherin or vimentin staining. After the staining procedure, the sections were mounted in fluorostab (Euro Diagnostica).

Image acquisition and analysis

The tissue sections were scanned with a digital image processing system consisting of a high-resolution 12-bit CCD camera (Micromax, Roper Scientific Inc., USA) on a fluorescence microscope (Axioskop, Zeiss, Germany) and a computer-controlled motorized stepping stage. Image processing was done using IPLab software (Scanalytics Inc., USA) on a Macintosh computer, as described earlier [17]. Each tissue section was sequentially scanned for all signals at 200x magnification. The resulting composite gray value images were converted to binary images for further analyses. Thresholds for the fluorescence signals were interactively set at intensities where the

steepest gradient occurred between signal to background intensity levels. The corresponding composite binary images were superimposed into one image for further image analysis. With help of an H&E staining of a consecutive section, the tumour area of each section was delineated. This area was used as a mask in further analysis from which non-tumour tissue; necrotic areas and artifacts were excluded. Vimentin expression in mesenchymal cells other than tumour cells (blood vessels, stromal components) was excluded from analysis. The fractions of expression of the markers were defined as the tumour area positive for the individual marker divided by the total tumour area.

Statistics

Statistical analyses were done on a Macintosh computer using Prism 4.0c (Hearne Scientific software, Ireland) software package. To determine correlations between parameters and categorical tumour characteristics (T-classification, N-classification, and differentiation-grade) χ^2–test, Spearman correlation and Kruskal-Wallis tests were used. To determine associations with distant metastasis formation, Kaplan-Meier graphs with log-rank testing were used. $p \leq 0.05$ was considered indicative of statistical significance.

Results

Patients and treatment

Twenty-eight patients were included in this study. Two biopsies were excluded from the analysis because they contained very little invasive carcinoma. Thus, 26 histologically confirmed HNSCC remained for analysis. Table 1 shows the clinical characteristics and treatment modalities of the patients. The median duration of follow-up was 25.9 months for all patients and 90.3 months for surviving patients.

Table 1. Patient and tumour characteristics of 26 HNSCC

Age	
Mean (range)	58 (36-80)
	Number (%)
Gender	
Male	23 (88)
Female	3 (12)
T-classification	
T2	7 (27)
T3	12 (46)
T4	7 (27)
N-classification	
N0	7 (27)
N+	19 (73)
Tumour site	
Larynx	12 (47)
Hypopharynx	5 (20)
Oropharynx	6 (24)
Oral cavity	2 (9)
Treatment	
Radiotherapy alone	14 (54)
Chemoradiation	5 (19)
Radiation + surgery	7 (27)

E-cadherin and vimentin expression in head and neck cancer

Staining of E-cadherin was limited to the cell membrane, while vimentin expression was observed in the cytoplasm. All markers gave bright fluorescent staining with little background except in areas of necrosis and stromal components of the tumour. E-cadherin expression was present in 96% (25/26) of the biopsies and was found throughout the tumour tissue in all samples (Figure 1). Vimentin expression was present in 46% (12/26) of the biopsies and was observed in tumour cells surrounding blood vessels (Figure 1). Sporadically, in three biopsies, we found vimentin expression in solitary tumour cells further away from blood vessels. No correlation was observed between overall expression of E-cadherin and vimentin in the different biopsies. No associations were found between expression of these markers and T-stage, N-stage or differentiation grade. Tumours with low E-cadherin and high vimentin fractions could identify tumours in which EMT has occurred (Figure 2A). In addition, no associations were observed for patients with low E-cadherin and high vimentin and T- and N-stage or differentiation grade.

Exploring the expression of E-cadherin and vimentin in relation to EGFR and pAkt might reveal associations between EMT and the EGFR-PI3-K/AKT pathway in patients with HNSCC. We observed tumour cells that show expression of E-cadherin, vimentin, EGFR and pAKT but also tumour cells without coexpression of more than one marker in the same tumour cell (Figure 1). We found a non-significant and weak association

between high E-cadherin fractions and high EGFR (r_s 0.28 p=0.18) and pAKT expression (r_s 0.27 p=0.19). Also, high vimentin expression was very weakly correlated with high EGFR (r_s 0.14 p=0.48) and not with pAKT fractions (r_s 0.02 p=0.9).

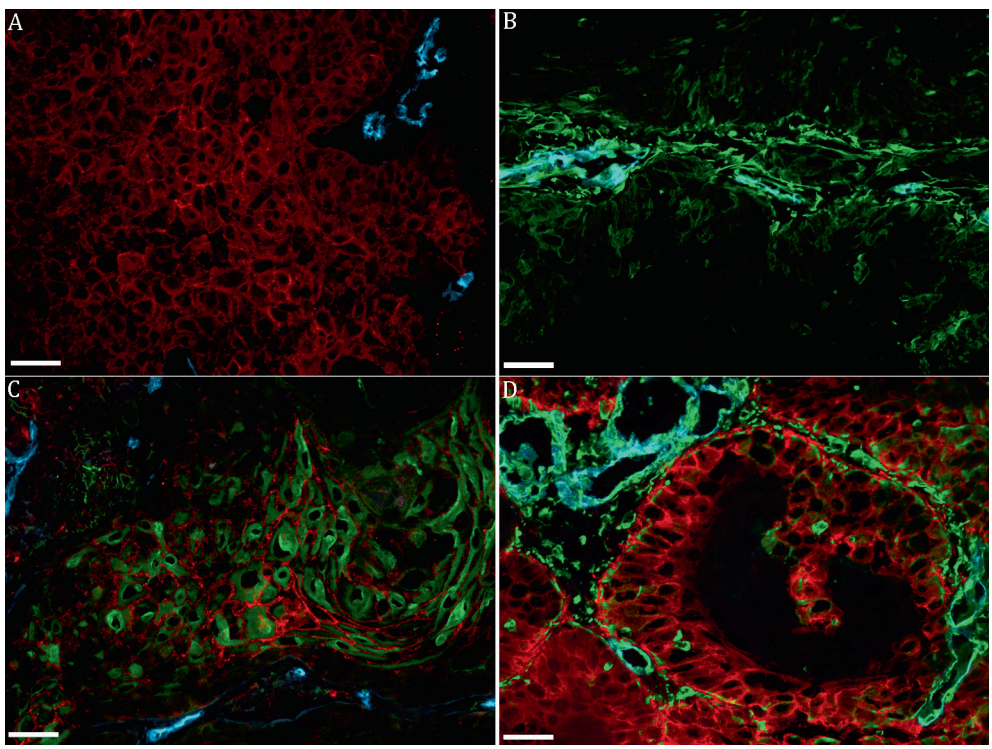

Figure 1. Fluorescence image at 200x magnification of (A) membranous E-cadherin (red), cytoplasmatic vimentin expression (green) and vessels (blue), (B) E-cadherin, pAKT (green) expression and vessels and (C) EGFR (red), vimentin expression and vessels. Bar represents 50 μm.

EMT and distant metastasis formation

Patients were dichotomized based on median expression values. A low expression of E-cadherin was significantly associated with a higher incidence of distant metastasis formation (p=0.004). The 5-year metastatic risk was 81% for patients with low E-cadherin expression versus 19% for patients with high E-cadherin expression (Figure 2B). Also, a high expression of vimentin showed a non-significant trend towards a higher metastatic risk (5-years risk 33% versus 77%, p=0.07) (Figure 2C). Figure 2D shows that patients with an EMT-phenotype (low E-cadherin and high vimentin

expression) have a significantly higher incidence of distant metastasis formation compared to the remaining patients (100% versus 44%, p=0.008). A further subgroup analysis is not realistic in view of the low total number of patients.

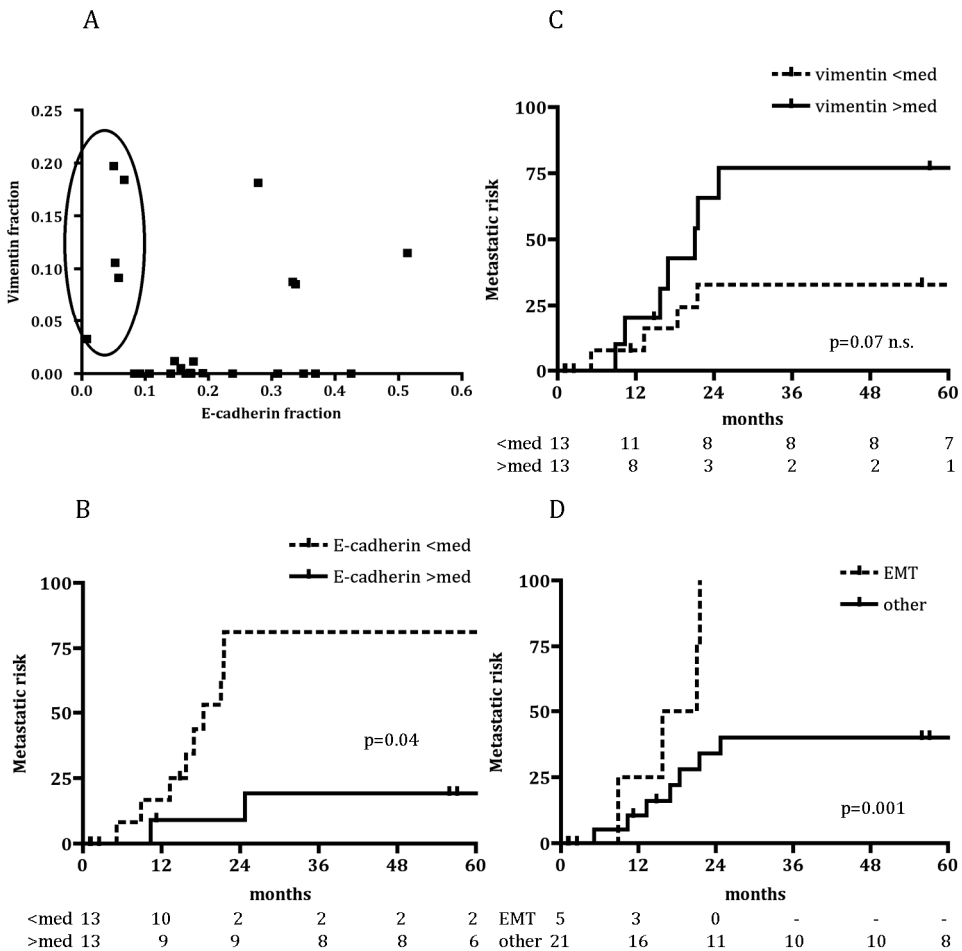

Figure 2. Distribution and correlation of E-cadherin and vimentin expression based on whole tissue sections in 26 head and neck biopsies. Tumours with an EMT-phenotype are encircled (A) and Kaplan-Meier estimates of metastatic risk of (B) E-cadherin expression, (C) vimentin expression (stratification by the median value) and (D) EMT-phenotype. Comparison by log-rank test. Numbers represent number of patients at risk.

Discussion

Changes in cell adhesion molecules have an important role in increasing the motility of tumour cells and thereby enhancing migration and the formation of metastasis. During EMT, epithelial cells transform and attain mesenchymal-like properties, such as loss of E-cadherin and gain of vimentin expression. Here, we investigated a heterogeneous (with regard to site, classification and treatment) group of head and neck cancer patients for expression of E-cadherin and vimentin. We found that loss of E-cadherin significantly correlated with increased risk of distant metastasis formation while increased vimentin expression showed a trend towards a correlation with this endpoint. Of the 26 patients included in our study, 21 patients received only local treatment, while five patients received chemotherapy in addition to radiation. Chemotherapy reduces the risk of metastatic failure. Therefore, we repeated the analysis excluding those patients (data not shown). This, however, did not lead to relevant differences in results or conclusions.

Previously, the expression of E-cadherin in primary carcinomas and nodal metastases of HNSCC and the relation to metastasis and patient survival has been explored [13,14]. The authors described more intense expression of E-cadherin in differentiated cells, but no correlation was found between reduced E-cadherin expression and survival (51 patients included in this study) [13]. This discrepancy in outcome might be explained by differences in staining techniques. Their group used the monoclonal antibody HECD-1, which detects the intracellular cytoplasmatic domain of the E-cadherin molecule, while a polyclonal antibody raised against the extracellular domain of E-cadherin was used in our study. A second study investigating E-cadherin expression and treatment outcome also showed no significant correlation with survival [14]. In contrast to our study, they dichotomized 57 patients in negative and positive for E-cadherin and correlated this only to overall survival, while we divided patient based on median values and looked for the incidence of distant metastasis formation.

Despite the fact that vimentin has been correlated with increased metastasis [4], it has not been extensively studied as prognostic marker in head and neck cancer treated with radiotherapy. Our results revealed that high vimentin expression shows a trend towards a higher incidence of metastasis formation. No correlation was observed between overall expression of E-cadherin and vimentin in the different biopsies, suggesting the presence of an intermediate phenotype with cells that passed only partly through the EMT. Although the numbers are small, we were able to identify a subset of tumours with low E-cadherin together with high vimentin fractions. These patients showed a significantly higher risk of metastasis formation compared to the tumours without this EMT-phenotype. A confirmatory study is currently being performed in a larger and more homogeneous cohort of patients with laryngeal carcinoma.

Overactivation of EGFR signalling pathways is related to more aggressive tumour behaviour and correlates with poor prognosis in patients with HNSCC [18-20]. Activation of the EGFR signalling cascades in turn can lead to transcription of genes responsible for cell cycle progression, cellular proliferation, DNA repair and metastasis [21,22]. EGFR signalling pathways are highly expressed in many human cancers, including carcinoma of the head and neck [23,24], leading to radioresistance and tumour progression [18,19,25]. Increasing evidence indicates that the EGFR signalling pathways can regulate expression of proteins involved in EMT in several tumour types [11,12]. This is supported by a study using a head and neck tumour model that highly expressed E-cadherin and that was very sensitive to the anti-EGFR antibody Cetuximab, whereas a tumour line that expressed vimentin revealed low sensitivity [26]. We observed that not all tumour cells that express E-cadherin also express EGFR although we found a weak association between high E-cadherin and high EGFR fractions. The relevance of this correlation for the prognosis of patients with head and neck cancer is not clear and could be due to the small number of patients.

The role of the EGFR down-stream target AKT in cell migration and metastasis is less clear. One study [27] showed that expression of AKT can induce EMT and promote enhanced motility and invasiveness in squamous cell carcinoma lines, while another study demonstrated that activation of AKT1 might suppress tumour invasion [28]. Irie et al described isoform-specific functions of AKT in the regulation of cell migration and invasion [29]. AKT1 down-regulation enhanced migration in response to EGF stimulation and induced an EMT phenotype: repressed E-cadherin expression and a small increase in vimentin expression. In contrast, AKT2 down-regulation does not enhance migration or alter expression of E-cadherin, but it does reduce vimentin expression.

In conclusion, our results show that a phenotype resembling EMT in patients with HNSCC, with loss of E-cadherin and gain of vimentin, is associated with a significantly higher risk of distant metastasis formation. If confirmed, this observation may have important implications for treatment decisions (e.g. (neo)adjuvant chemotherapy).

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Chapter 6

Low pAKT expression in laryngeal cancer; indications for a higher metastatic risk.

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Abstract

The PI3-K/AKT pathway plays an important role in tumour cell survival and radioresistance. Targeting AKT is considered as a treatment option in several solid tumour types. Recently; however, we found in head and neck cancer patients that low phosphorylated (p)AKT expression in the primary tumour was associated with lymph node metastasis. Here, we set out to validate this finding in an independent cohort of laryngeal cancer patients and to examine the effect of pAKT inhibition on epithelial-mesenchymal transition (EMT) of laryngeal cancer cells.

Seventy-eight patients with laryngeal cancer were included. EGFR, pAKT, vimentin, E-cadherin, hypoxia and blood vessels were visualized in biopsy material using immunohistochemistry. Positive tumour areas and spatial relationships between markers were assessed by automated image analysis. In six laryngeal cancer cell lines E-cadherin and vimentin mRNA was quantified by real-time polymerase chain reaction and by immunohistochemistry before and after treatment with the pAKT inhibitor MK-2206.

A significant correlation was found between low pAKT in the primary tumour and positive lymph node status (p=0.0005). Tumours with lymph node metastases had approximately 10-fold lower median pAKT-value compared to tumours without lymph node metastases, albeit with large inter-tumour variations, validating our previous results. After inhibition of pAKT in laryngeal cancer cells with MK-2206 upregulation of vimentin and a downregulation of E-cadherin occurred, consistent with EMT.

Low pAKT expression in larynx tumours is associated with lymph node metastases. Further, inhibition of pAKT in laryngeal cancer induces EMT predisposing for an increased metastatic risk.

Introduction

Radiotherapy is an effective treatment modality for head and neck cancer, in particular for early-stage tumours. In advanced stages the effectiveness of radiotherapy has been improved by the addition of chemotherapy or the Epidermal Growth Factor Receptor (EGFR) inhibition by cetuximab [1,2]. Currently, the combination of radiotherapy with inhibitors of pAKT, a signaling molecule downstream of EGFR, is under clinical investigation. However, recent findings showed an association of low pAKT with increased metastases formation in patients with head and neck cancer treated with radiotherapy [3]. This raises the question if pAKT inhibition in head and neck cancer may actually promote a more metastatic phenotype leading to a worse clinical outcome.

AKT, a serine/threonine protein kinase, is one of the most frequently hyperactivated signaling pathways in human cancers. It is phosphorylated by phosphatidylinositide-3-kinase (PI3-K) after activation through receptor tyrosine kinases (RTK) like EGFR. After phosphorylation at the plasma membrane AKT translocates to the cytosol and nucleus to activate its substrates [4]. Although the main known biological consequences of AKT activation are proliferation, growth and tumour-induced angiogenesis, the prognostic significance of AKT activation in cancer is inconclusive. High pAKT expression has been associated with poor [5] and favorable prognosis [6] in different types of tumours.

Three isoforms of AKT exist, of which AKT1 and AKT2 are ubiquitously expressed. All are activated by similar mechanisms in PI3-K signaling [7]. The different isoforms of AKT all have cancer-type specific roles in cell migration and metastasis formation. In prostate cancer cells, both AKT1 and AKT2 function as negative regulators of cell migration and invasion. Downregulation of AKT1 but not AKT2 in breast cancer cells caused an enhancement of cell migration [8]. This enhanced migration was accompanied by phenotypical changes that are consistent with the phenotypical change of epithelial-mesenchymal transition (EMT). In EMT, the expression of epithelial markers (E-cadherin) is suppressed and the expression of mesenchymal markers (vimentin) is enhanced, leading to reduced cell-cell adhesion and possibly increased cell migration. Earlier investigations show more metastases and a reduced patient survival when tumour cells exhibit a more mesenchymal phenotype [9,10]. MK-2206, currently used in clinical trials, is an allosteric inhibitor of AKT phosphorylation [11].

The purpose of this study was to validate the association of pAKT with lymph node metastasis in an independent, homogeneous cohort of patients with larynx cancer. Furthermore, we studied the effect of MK-2206-induced pAKT inhibition on the expression of two major proteins involved in EMT, E-cadherin and vimentin, in laryngeal cancer cell lines.

Patients and methods

Patients

Between April 2001 and January 2008, 78 patients with squamous cell carcinoma of the larynx were included. All patients were treated in the Radboud University Nijmegen Medical Centre Nijmegen, The Netherlands. The study was approved by from the local ethics committee and all patients provided written informed consent. Patients were treated with (accelerated) radiotherapy alone or surgery with post-operative radiotherapy. Approximately 2 h before taking a biopsy, patients received a 20 min intravenous (i.v.) infusion of the hypoxia marker Hypoxyprobe-1 (500 mg/m^2) (pimonidazole-hydrochloride; NPI Inc.). Biopsies were taken for routine diagnostic purposes and additional biopsies were taken for multiple marker analyses. The latter were snap frozen in liquid nitrogen.

Immunohistochemistry

Frozen tumour sections (5 μm) were mounted on poly-L-lysine coated slides and stored at -80°C. Next sections were fixed in cold acetone (4°C, 10 min) and rehydrated in phosphate buffered saline (PBS Klinipath, The Netherlands). Between all steps of the staining procedure, sections were rinsed in PBS three times. Sections were incubated overnight at 4°C with rabbit anti-pAKT antibody (Ser473) and goat anti-EGFR antibody diluted 1:50 in primary antibody dilution (PAD, GeneTex Inc.). Adjacent sections were incubated overnight with rabbit anti-pimonidazole antibody (J.A. Raleigh, University of North Carolina, USA) and goat anti-EGFR antibody (1:1000 and 1:50 in PAD respectively). A third adjacent tumour section was incubated with goat anti-E-cadherin 1:50 in PAD. For all sections, the second incubation was with donkey anti-rabbit Alexa488 (Molecular Probes, The Netherlands) or donkey anti-goat Cy3 (Jackson Immunoresearch Laboratories Inc.) diluted 1:600 in PBS for 30 min at 37°C. The sections were stained for vessels by incubation with the mouse anti-human endothelium antibody PAL-E (Euro Diagnostica, The Netherlands) (1:10 in PAD) followed by incubation for 30 min at 37°C with chicken anti-mouse Alexa647 antibody (Molecular Probes) (1:100 in PBS). Next, sections stained for E-cadherin were incubated with rabbit anti-vimentin 30 min (room temperature, 1:200 in PAD) followed by incubation for 45 min with donkey anti-rabbit Alexa488 1:100 in PAD. Antibodies were purchased from Santa Cruz Biotechnology Inc., CA. After the staining procedure, the sections were mounted in fluorostab (ProGen Biotechnik GmBH, Germany). UT-SCC cells cultured on chamberslides were stained similarly.

Image acquisition

The tumour sections were scanned with a digital image processing system on a fluorescence microscope (Axioskop, Zeiss, Germany) and a computer-controlled motorised stepping stage. Image processing was done using IPLab (Scanalytisc Inc.)

and ImageJ software (National Institute of Health, USA) on a Macintosh computer, as described earlier [12,13]. Each section was sequentially scanned for all signals at 200X magnification. The resulting composite grey value images were converted to binary images for analyses. Thresholds for the fluorescence signals were interactively set at intensities where the steepest gradient clearly distinguishing between signal and background. The corresponding composite binary images were superimposed into one pseudo-coloured image for visual evaluation.

Analysis

With H&E staining of a consecutive section, the tumour area of each section was delineated. This area was used as a mask in further analysis from which non-tumour tissue; necrotic areas and artefacts were excluded. Vimentin expression in mesenchymal cells other than tumour cells (blood vessels, stromal components) was excluded from analysis. The fractions of marker expression were defined as the tumour area positive for the individual marker divided by the total tumour area.

To quantify the distribution of hypoxia, EGFR and pAKT in relation to the vasculature, zones were chosen at increasing distances from the nearest vessel (0-50, 51-100, 101-150, 151-200, and >201 μm). Hypoxic fractions, as well as fractions EGFR and pAKT were calculated within these vascular zones.

Cell culture

Six human laryngeal cancer cell lines (UT-SCC lines, University of Turku, Finland) were cultured *in vitro*. All patients from whom the cell lines were derived were N0 except UT-SCC9, which was N1. Cells were cultured under humidified conditions (37°C, 5% CO_2), and passaged twice weekly in DMEM containing 2mM L-glutamine, 1% nonessential amino acids, 20 mM Hepes, 10 units/ml penicillin, 10 units/ml streptomycin and 10% foetal bovine serum. To determine the effect of low pAKT levels in tumours, cell lines were treated overnight with 1 μM of the pAKT inhibitor MK-2206 (Selleckchem, Houston, USA) under standard normoxic conditions.

qPCR

To determine the effect of pAKT inhibition on EMT, cells were lysed, RNA isolated, and E-cadherin and vimentin mRNA quantified using qPCR. Total RNA was isolated with total RNA purification kit (Norgen Biotek Corp., Canada) with on-column DNAse treatment. RNA was reversed-transcribed using I-script (Bio-Rad) and cDNAs were amplified with specific primers (E-cadherin forward: AGGCCAAGCAGCAGTACATT, reverse: ATTCACATCCAGCACATCCA; Vimentin forward: ACACCCTGCAATCTTTCAGACA, reverse: GATTCCACTTTGCGTTCAAGGT) using Sybr Green Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) on a CFX96 realtime-PCR detection system (Bio-Rad Laboratories Inc. Richmond, CA). All

samples were normalized for levels of hypoxanthine-guanine phosphoribosyl-transferase (HPRT) expression.

Western blot

To verify if pAKT expression was inhibited by MK-2206 cells were lysed, debris was removed and protein was quantified using a standard Bradford absorbance assay. Proteins were separated by SDS-PAGE and blotted onto PVDF membrane. Membrane was incubated with a pAKT (Ser473) antibody (Cell Signaling Technology, Danvers, MA) followed by incubation with HRP-conjugated antibody goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, USA) and detected with an ECL chemiluminescence system. Protein quality and loading check was performed with α-tubulin (Calbiochem San Diego, CA)

Statistics

Statistical analyses were done on a Macintosh computer using Prism 4.0c (Hearne Scientific software, Ireland) software package. To determine correlations between parameters and categorical tumour characteristics (T-classification, N-classification and differentiation grade) Spearman correlation and Kruskal-Wallis tests were used.
$p \leq 0.05$ was considered statistical significant.

Results

Patients

A total of 78 patients were included in this study. Pimonidazole was given to all patients before biopsy taking and none of them had adverse reactions. Nine biopsies were excluded from analysis, three because they contained no or little invasive carcinoma, four because of poor staining quality and two because histological diagnosis was not laryngeal carcinoma. Thus, 69 histological confirmed laryngeal carcinomas were used for analysis (Table 1).

Table 1. Patient and tumour characteristics (N=69)

Age	
Mean (range)	61 (38-83)
	Number (%)
Gender	
Male	50 (72)
Female	19 (28)
T-classification	
T2	29 (42)
T3	27 (39)
T4	13 (19)
N-classification	
N0	42 (61)
N+	27 (39)
Tumour site	
Glottic	20 (29)
Supraglottic	43 (62)
Subglottic	2 (3)
Transglottic	4 (6)
Diff. grade	
Good	5 (7)
Moderate	41 (60)
Poor	18 (26)
Not classified	5 (7)

EGFR, pAKT, E-cadherin & vimentin expression in larynx carcinoma

Staining of EGFR and E-cadherin was limited to the cell membrane, while pAKT and vimentin expression was observed in the cytoplasm (Figure 1). As expected, pimonidazole binding increased at increasing distances from the blood vessels with highest fractions at >150 μm from the nearest vessel. Expression of EGFR was predominantly found in better-oxygenated areas close to blood vessels, while pAKT expression was higher in pimonidazole positive, hypoxic areas (Figure 2).

There was a strong significant negative correlation between pAKT expression and N-stage (p=0.0005). Laryngeal carcinomas with lymph node metastases had much lower pAKT expression compared to tumours with negative lymph node (median fraction 0.08 versus 0.009, Figure 3). No further associations were found between expression of EGFR or pAKT and other clinical parameters.

Previously, we hypothesized that pAKT attenuates the transition of epithelial cells to a more mesenchymal phenotype, thereby explaining lymphatic invasion specifically in pAKT negative tumours. We therefore explored E-cadherin and vimentin expression in relation to pAKT. Some pAKT positive cells were also positive for E-cadherin, while others coexpressed pAKT and vimentin.

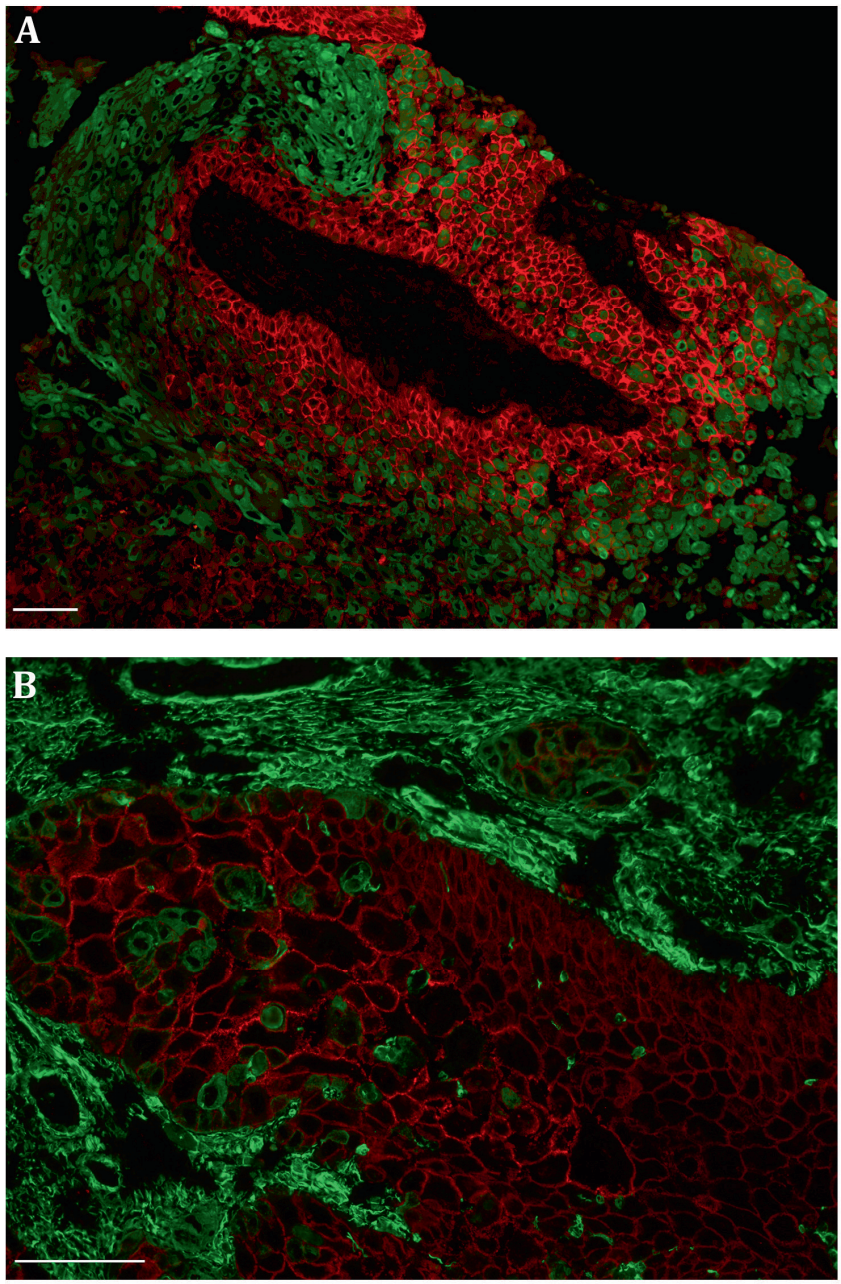

Figure 1. Immumofluorescene image of a biopsy of a laryngeal carcinoma showing (A) membranous EGFR staining (red) and cytoplasmatic pAKT staining (green) (100X magnification) and (B) membranous E-cadherin staining (red) and cytoplasmatic vimentin (green) (200X magnification). Scalebars represent 100 μm.

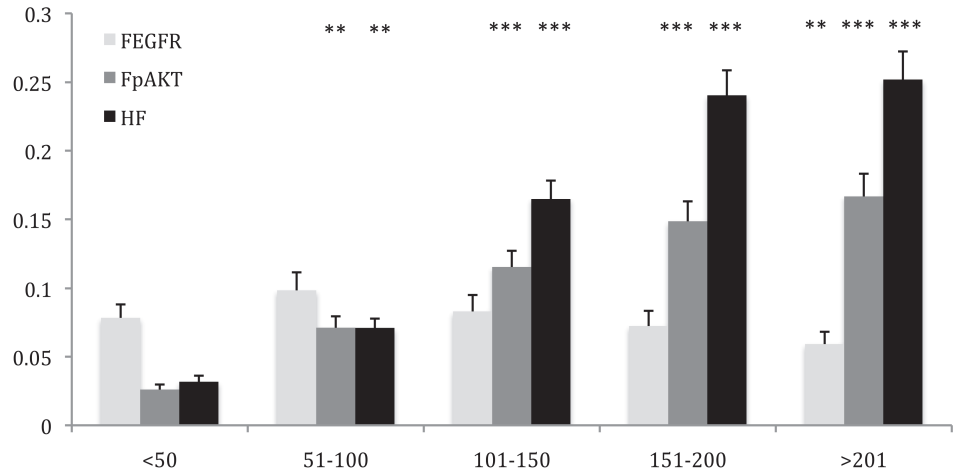

Figure 2. Distribution of pimonidazole (black), fraction EGFR (light gray) and pAKT (dark gray) as a function of distance (μm) from nearest vessel. Average values of 69 tumours (± SEM). ** p<0.01 *** p< 0.0001 significant difference compared to <50μm zone.

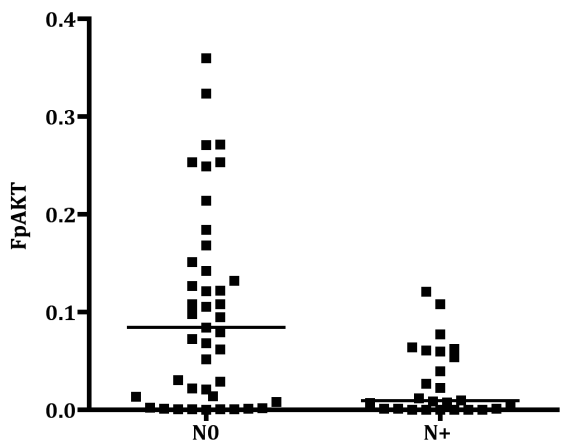

Figure 3. Expression of pAKT in node positive (N+) versus node negative (N0) tumours. Lines represent median values. p=0.0005

pAKT inhibition leads to reduced E-cadherin and upregulation of vimentin in larynx tumour cells

To place the results from the patient data in context, we examined if inhibition of pAKT with MK-2206, a novel allosteric pAKT inhibitor, would induce EMT in laryngeal carcinoma cells. We found that pAKT inhibition after 24 or 48 hr incubation upregulated vimentin mRNA in all six cell lines, although not all differences were statistically significant. In four cell lines mRNA of E-cadherin was reduced (Figure 4A.). Two cell lines, UT-SCC 9 and UT-SCC 29, downregulated E-cadherin and upregulated vimentin mRNA within 24 hr of pAKT inhibition, which is a classical indication of EMT. All other cells lines showed a non-significant trend towards EMT. Figure 4B shows that treatment with 1 μM MK-2206 inhibited expression of pAKT assessed by the means of western blotting.

To investigate the effect of pAKT inhibition on protein expression of EGFR, E-cadherin and vimentin we immunohistochemically stained these markers in UT-SCC 9 cells. This tumour cell line, showing an EMT-like transition with qPCR (Figure 4A), was cultured on chamberslides. Figure 4C&D clearly shows that after pAKT inhibition a downregulation of E-cadherin expression and an upregulation of vimentin expression occur. Unexpectedly, we found expression of nuclear EGFR after treatment with MK-2206 (Figure 5).

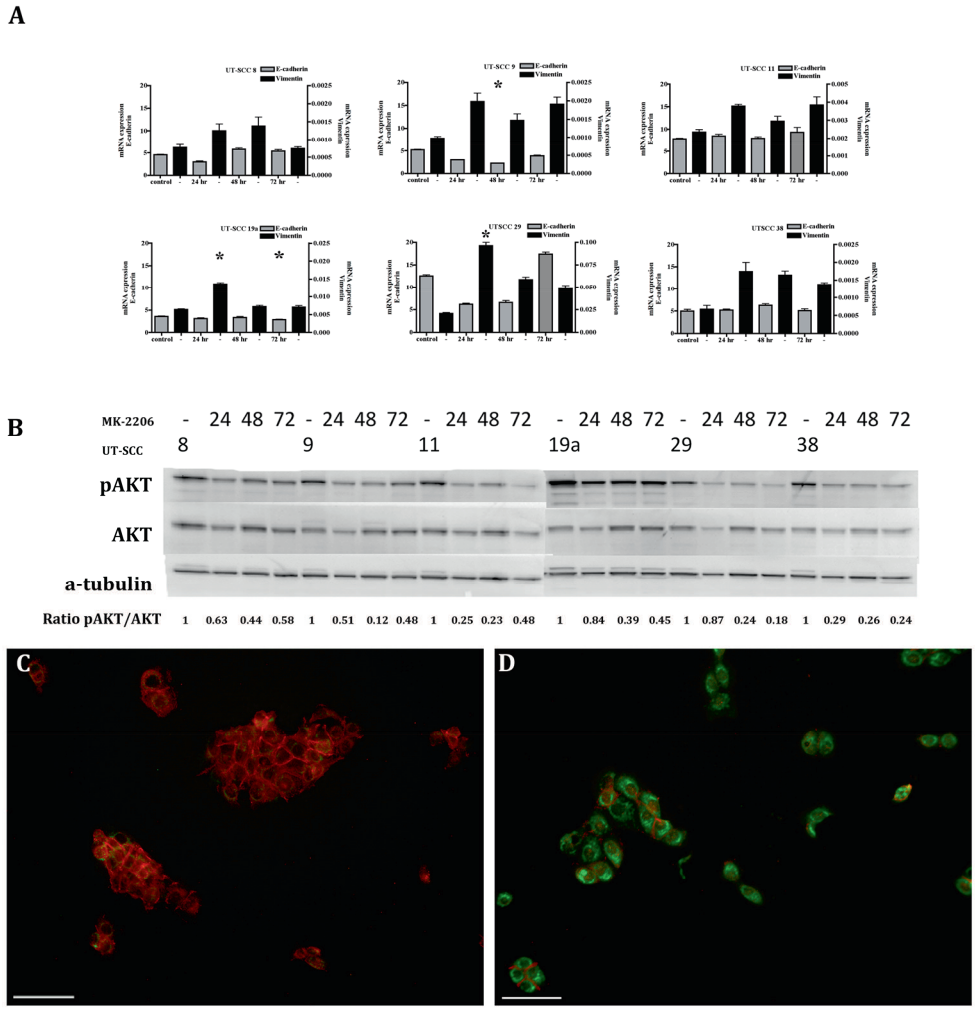

Figure 4. mRNA levels of Ecadherin and vimentin relative to HPRT after 24, 48 or 72 hr pAKT inhibition (A). *Represent significant difference compared to control cells. Downregulation of pAKT was shown by Western Blot analysis; numbers below the bands are densitometry values of pAKT normalized against AKT values (B). Fluorescence images (200X magnification) of E-cadherin (red) and vimentin (green) before (C) and after (D) pAKT inhibition. Scalebars represent 100 μm

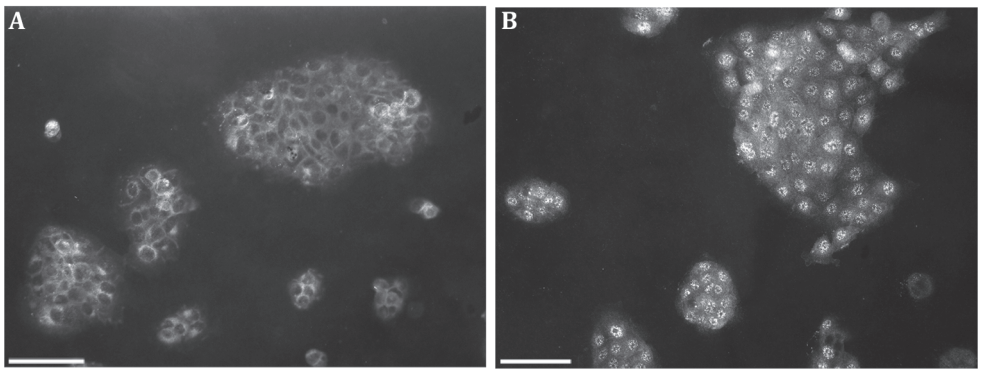

Figure 5. UT-SCC cells after treatment (B) with MK-2206 for 24 hr an upregulation of nuclear EGFR was present, control cells showing membranous EGFR (A). Magnification 200X, scalebars represent 100 μm.

Discussion

Anti-EGFR targeted therapy in combination with radiotherapy has been shown to improve survival but only in a small percentage of patients with head and neck cancer [2]. Therefore, new targets are tested for their usefulness as treatment options. The PI3-K/AKT pathway is a downstream effector of EGFR, and AKT is an interesting candidate for therapeutic purposes because, like EGFR, it is often overexpressed in HNSCC [14]. However, we predominantly found EGFR expression in better-oxygenated areas of the tumour, while pAKT levels were higher in hypoxic areas. This finding supports the evidence that not only EGFR, but multiple receptors can activate AKT. It also points out the need for improved targeted therapies, as EGFR inhibition does not automatically lead to inhibition of AKT-dependent survival mechanisms.

Although it is generally accepted that activation of AKT leads to enhanced tumour growth and poor outcome, preclinical studies indicate that low AKT1 expression can lead to more metastases [8]. Recently, we found an association between low pAKT expression and positive nodal status of HNSCC patients prior to treatment [3]. We speculated that pAKT inhibition could stimulate metastasis formation in these tumours. Here, we have validated this observation in an independent cohort of laryngeal cancer patients. Patients presenting with lymph node metastases appear to have 10-times lower median pAKT expression levels in their primary tumour compared to patients without nodal metastases, although there are large variations between tumours and an overlap between both groups exists. This further supports the evidence that AKT is associated with metastatic prevalence of HNSCC.

Different AKT isoforms are found to have distinct functions in tumour progression depending on tumour type. Enhanced AKT1 signaling promotes tumour progression through increased cellular survival mechanisms, whereas AKT2 reportedly inhibits

cancer growth [15]. AKT1 reduces while AKT2 enhances cell invasion and migration in breast and ovarian cancer but, on the other hand, no different isoform specific functions were found in prostate cancer [8,15,16] This indicates that treatment with pAKT inhibitors, aimed at both isoforms together, might inhibit growth of some tumours, but could also induce more pronounced metastatic behavior in others. These findings highlight the necessity for isoform-specific inhibitors. For example, AKT1 inhibition may not be recommended in breast and, maybe, in head and neck cancer patients, as it may promote cell migration, whereas in other cancer types, such as prostate cancer, all AKT inhibition may be beneficial to inhibit tumour progression.

Also, *in vivo*, there are contradicting results on the role of AKT in metastasis formation. In mice with mammary tumours overexpressing ErbB2 AKT1 coexpression impaired metastases while AKT2 coexpression increased the proportion of mice with metastasis [8,17]. Inhibition of pAKT with MK-2206 in an orthotopic model of a tongue tumour led to reduced primary tumour size and less cervical metastases [11]. When testing different clonal mammary tumour cell lines Dillon et al. found that highly metastatic clones displayed upregulated AKT2 expression compared to less metastatic clones. Interestingly, the highest pAKT levels, all due to elevated AKT1 phosphorylation, were found in the low metastatic clones [17]. The data presented in the current study also showed a higher pAKT level in tumours without lymph node metastasis.

Although downstream effectors of AKT isoforms remain to be identified, candidate pathways have been suggested. One study found that AKT1 could inhibit breast cancer cell invasion through nuclear factor of activated T-cell (NFAT) downregulation. Another study showed that the enhanced migration observed with AKT1 downregulation was accompanied by changes in protein expression that are consistent with EMT [8]. Metastasis formation not only involves EMT, but also detachment from the primary tumour site and escape of single cells into the blood or lymph vessels followed by reattachment, transition back to epithelial state and angiogenesis to form a secondary tumour [9]. We hypothesized that pAKT can protect cells from EMT. Therefore, reducing pAKT in tumour cells should lead to specific up or down regulation of proteins involved in EMT. In six laryngeal carcinoma cell lines we tested whether reducing pAKT with MK-2206, a pAKT inhibitor, leads to downregulation of E-cadherin and upregulation of vimentin. In all cell lines EMT was induced to some degree, indicating that inhibition of pAKT in laryngeal tumour cells possibly leads to a higher metastatic risk.

Another explanation why low pAKT can lead to more metastases could be the existence of a negative feedback loop. Recently was found that inhibition of mTOR by rapamycin relieves a feedback loop, activating IGF signaling leading to activation of PI3K and ERK signaling. This was found also in patients thereby decreasing the therapeutic efficacy of the drug [18]. Others provided evidence that inhibition of AKT induces HER3 expression and other RTKs by a similar feedback loop. By inhibition of

AKT the downstream effects will be suppressed but other RTKs-driven signaling pathways, like mTOR and MDM2, will be activated, leading to activation of proteins and genes involved in migration of tumour cells, explaining the effect of low pAKT expression on metastatic potential [19].
An unexpected finding after pAKT inhibition was the induction of nuclear EGFR. EGFR is predominantly present on the cell membrane, however recently nuclear localisation of EGFR has been identified. Nuclear EGFR was found to act as transcriptional activator of various oncogenic genes; it is associated with increased G1/S phase progression and proliferation of tumour cells. Nuclear EGFR correlates with poor outcome in patients with breast, oropharyngeal and ovarian cancer [20]. Also, downregulation of pAKT induces RTK activity including EGFR and HER3 in breast cancer cells but no distinction was made between nuclear and membranous EGFR [19]. In the present study we could clearly see in which subcellular compartment EGFR is upregulated. Whether this MK-2206-induced nuclear EGFR is a tumour-specific effect of laryngeal cancer and how this affects radioresistance requires further investigation.

Conclusion

Several ongoing clinical trials use pAKT inhibitors to modulate treatment response. Inhibition of pAKT is expected to lead to a better patient outcome by reducing downstream signaling of tumour survival mechanisms. The results from our work and studies by others describe a complex picture in which pAKT inhibition should be considered in a cancer-type specific manner. We recommend that new pAKT inhibitors should be tested for potential stimulation of EMT and be introduced in the clinic prudently.

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Chapter 7

General Discussion

Discussion

Due to a preference for organ preservation, the first choice of treatment of early-stage tumours is often radiotherapy. Tumour cell response to radiation depends on activation of different receptor proteins and signalling pathways; on differences in intrinsic radioresistance, tumour cell proliferation and the amount of hypoxic regions within a tumour [1]. All these aspects are, to some extent, under the control of the EGFR-PI3-K/AKT signalling pathway. The aim of this thesis was to investigate the activation of this pathway, which is often overexpressed in solid tumours including head and neck squamous cell carcinomas (HNSCC), the tumour model that was used in this thesis. In order to understand the involvement of this pathway in radiation responsiveness and in an attempt to disentangle the interactions with the tumour microenvironment, we evaluated expression levels of key molecules in relation to each other and to microenvironmental factors, like the presence of hypoxia and vasculature. In this chapter, the relevance of the EGFR-PI3/K-AKT pathway for radiation responsiveness is discussed and future considerations are highlighted.

EGFR and downstream signalling pathways

EGFR and its downstream signalling pathways have been frequently investigated with regard to patient outcome [2-4]. The current knowledge on EGFR-regulated signalling pathways provides strong evidence that EGFR activation plays an important role in the regulation of tumour cell survival and treatment resistance. EGFR is a transmembrane protein with intrinsic tyrosine kinase activity [4] and activation of EGFR causes autophosphorylation (pEGFR), subsequent receptor internalisation and stimulation of many signalling pathways including the phosphatidylinositol-3-kinase-AKT (PI3-K/AKT) pathway [1,5,6]. Once activated, these pathways are responsible for tumour cell proliferation, DNA-damage repair, migration, angiogenesis, and consequently resistance to treatment. Radiotherapy combined with blockage of EGFR by cetuximab has resulted in improved locoregional control and survival for patients with HNSCC [7] demonstrating that EGFR is a clinically relevant target for molecular therapies in addition to radiation.

Tumour microenvironment

Understanding the regulation of signalling pathways in tumours insensitive to particular treatment regimens will give information on tumour behaviour and can improve selection of patients for customised treatment options. A malignant tumour not only contains cancer cells but also exists of a variety of normal cell types that interact with each other and with a microenvironment that is characterised by both temporal and spatial heterogeneity. An imbalance between oxygen consumption and supply will result in (temporal) hypoxic tumour areas. Within the tumour microenvironment, hypoxia is relevant in almost all solid tumours. In our lab, research

regarding EGFR and tumour oxygenation is based on hypoxia measurements by pimonidazole staining [3,8,9]. Pimonidazole is a robust exogenous marker of hypoxia [10] and it only detects viable, hypoxic tumour cells, because a reduction step and binding are necessary for immunohistochemical detection. Several studies have proven the relevance and predictive value of pimonidazole [11-14]. Results from clinical trials with a treatment that counteracts enhanced tumour cell proliferation as well as hypoxia (Accelerated Radiotherapy with CarbOgen and Nicotinamide (ARCON)) show high locoregional control rates, in particular for oropharynx and larynx carcinoma patients with high pimonidazole binding levels [11,15].
As described in this thesis, EGFR expression was predominantly found in better-oxygenated tumour areas. Further, based on *in vitro* experiments, it is generally thought that EGFR and pAKT expression colocalize in the same tumour cells. However, it was surprising to find that in hypoxic areas AKT is activated without EGFR expression. This is a good example of the important role of the tumour microenvironment being responsible for the activation of the AKT protein, independent of EGFR. This indicates that the concept of EGFR-induced AKT activation is a simplification of a complex interaction between signal transduction pathways and the tumour microenvironment.
Not only EGFR but also other receptors and mechanisms can activate AKT in more hypoxic areas. These include activation by other ErbB family members or receptor kinases like VEGFR [16], amplifications or mutations of the gene *PIK3CA*, amplifications of AKT itself or decreased expression of PTEN, a tumour suppressor [17-20]. On the other hand, (p)EGFR expression without pAKT expression as seen in some normoxic areas, indicates that not in all cells EGFR is actually activated or that EGFR activation necessarily leads to activation of the downstream PI3-K/AKT pathway. Apart from activation of AKT, EGFR can activate other cell survival pathways including mTOR and RAS/RAF/MAPK [16]. Therefore, future research should focus on these questions; how is AKT activated in hypoxic areas and can we target this activation upstream of AKT? And, apart from PI3-K/AKT, which EGFR-dependent pathways are mostly activated in normoxic areas and is there a link with radiotherapy resistance?

Consequences for radiotherapy
The amount of EGFR expression in a tumour is relevant for radiation response and is a prognostic factor after conventional fractionated radiotherapy [2] and surgery [21,22]. In the earlier 90's two randomised trials showed that acceleration of radiotherapy schedules could improve patient outcome of high EGFR expressing tumours to the level of low EGFR expressing tumours, presumably by counteracting enhanced tumour cell proliferation [23,24]. In chapter 3 of this thesis we demonstrate that EGFR is predictive for the response to accelerated radiotherapy as well as for the response to hypoxia modification but in reverse ways: high EGFR expression levels

predict for good response to accelerated radiotherapy whereas low EGFR expression levels predict for good response to hypoxia modification.
It is known that EGFR signalling is associated with the hypoxia response of tumour cells. Blocking EGFR can reduce intratumoural hypoxia by normalization of the irregular dysfunctional tumour vasculature, thereby improving perfusion and oxygen delivery [25-27] or by improved reoxygenation during fractionated radiotherapy [28]. A major pathway in the hypoxia response is the HIF-pathway. Under normoxic conditions HIF-1 is rapidly degraded, but under hypoxia it is stabilized [29-31]. Tumour cells with high pre-treatment EGFR expression levels might be better able to rapidly activate downstream survival pathways, resulting in activation of the HIF-pathway, thereby thwarting the hypoxia modifying effect of ARCON. Possibly, therefore no advantage of this treatment was found for patients with high EGFR expressing tumours. In the latter, the role of EGFR inhibition in combination with hypoxia modification needs to be explored. By adding cetuximab, a monoclonal antibody against the EGF receptor, or erlotinib or gefitinib, EGFR tyrosine kinase inhibitors, EGFR signalling can be inhibited before treatment with ARCON. Combining these modalities for patients with high EGFR expressing tumours with high amount of hypoxic regions might be a next step forward in individualised treatment schedules leading to better survival for cancer patients.

Different faces of AKT
Anti-EGFR targeted therapy in combination with radiation has indeed shown to improve survival but only in a minor percentage of the patients (15% at best) [7]. New targets are tested for their usefulness as treatment options including AKT. Currently, the combination of radiotherapy with inhibitors of pAKT is under clinical investigation. Although the main known biological consequences of AKT activation are proliferation, growth, and tumour-induced angiogenesis, the prognostic significance of AKT activation in cancer is inconclusive. High pAKT expression has been associated with poor [32-35] and favourable prognosis [36-39] in different types of tumours. As presented in chapters 4 and 6 of this thesis, we found an association of low pAKT expression with increased regional metastatic risk in head and neck patients treated with radiotherapy. This raises the question if pAKT inhibition in head and neck cancer may actually promote a more metastatic phenotype and can lead to a worse clinical outcome. Three distinct genes encode for three isoforms of AKT. There are indications that these different isoforms have distinct functions in tumour progression depending on tumour type. Enhanced AKT1 signalling promotes tumour progression through increased cellular survival mechanisms, whereas AKT2 reportedly inhibits cancer growth [40-42]. AKT1 reduces while AKT2 enhances cell invasion and migration in breast and ovarian cancer [43,44]. Treatment with pAKT inhibitors, aimed at both isoforms together, might inhibit growth in some tumours, but could also induce a more pronounced metastatic behaviour in others. Unfortunately, we were not able to

perform AKT iso-form specific immunohistochemistry on our head and neck tumours, but in subsequent research this will be a major aim. The findings also highlight the necessity for isoform specific inhibitors and the importance of testing new molecular inhibitors for potential reverse outcome on different endpoints (local control versus metastases formation) before being introduced in the clinic.

In this context it is noteworthy that in two independent cohorts of head and neck cancer patients, the second being a validation of the first study, we have found that low pAKT expression in primary tumours has a reverse correlation with lymph node metastases. Patients with negative lymph nodes had a significantly higher expression of pAKT compared to patients with positive lymph nodes. We hypothesized that low pAKT expression could lead to more migratory tumour cells through the process of epithelial to mesenchymal transition (EMT) [45]. In chapter 5 we showed that this EMT phenotype, characterized by reduced E-cadherin and upregulation of vimentin expression [46], is associated with a higher metastatic risk. In addition, when using a pAKT inhibitor laryngeal tumour cells reduced their E-cadherin expression and upregulated the expression of vimentin. With new techniques in our laboratory, e.g. migration and invasion assays, we now should be able to perform experiments thereby answering the question whether low pAKT and subsequently EMT leads to more metastases in our head and neck tumour models [47].

Another issue is whether lymph node metastases have the same expression levels of pAKT as the primary tumour. Is AKT only protecting tumour cells to undergo EMT and to migrate or does AKT maybe have a different role in tumour progression at the secondary sites? These questions are important for further understanding the influence of AKT in metastatic risk. Unfortunately, for the patients investigated in this thesis no tissue samples of the corresponding lymph nodes were available. In a new cohort of patients, biopsies of primary tumours and the corresponding metastatic lymph nodes will be collected, making it possible to further investigate these questions.

Immunohistochemistry on biopsy material: strengths and limitations

To demonstrate protein expression levels in biopsies of head and neck cancer patients, immunohistochemistry was used in this thesis. A major advantage of this method is that the amount of different proteins, their subcellular location and the expression relative to each other or to hypoxic areas and vessels with preservation of the tissue architecture can be investigated. In chapter 4 the co-expression of activated EGFR (pEGFR) and AKT (pAKT) is assessed relative to the vasculature. Although a positive correlation between total fraction pEGFR and pAKT was observed, levels of co-expression were low. Interestingly, the highest fractions of co-expression was close to the vessels, which again indicates that in head and neck tumours EGFR is only

partly responsible for activation of the PI3-K/AKT pathway. In more hypoxic areas AKT seems to be activated in an EGFR-independent manner. As these hypoxic tumour cells are able to activate proteins like AKT under harsh circumstances they may form an important subpopulation of the tumour responsible for tumour progression and treatment failure. Knowledge about the precise role and interaction of these proteins gives more insight in tumour cell behaviour and radioresistance.

A point of concern is that biopsies were used which represents only part of the entire tumour. As a tumour is a heterogeneous interaction between cells and their environment, it is possible that valuable information is missed when taking a biopsy. This tumour heterogeneity was demonstrated in a study measuring proliferation in the centre and from the edge of an oesophageal carcinoma. There was a significant difference in proliferation index between these sites and this heterogeneity should be taken into account when using biopsy material [48].
To be able to identity thousands of genes and their expression patterns simultaneously, microarrays have been developed. Tumour response to radiotherapy might be predicted by identification of genes that are differentially expressed between radiosensitive and radioresistant tumours. Although these gene expression profilers can provide insights in genes involved in treatment failure, they are relatively expensive, difficult to interpret in a clinical setting, and statistics are complex. In addition, the microarrays provide no information about the expression pattern of the gene in relation to important microenvironmental features such as tumour cell hypoxia and vasculature. A combination of gene expression profiling and immunohistochemistry would be a valuable tool to gain more insight in which genes are involved in radioresistance [49,50]
Characterisation of the whole tumour is possible with PET/CT or MRI techniques prior, during, and after treatment. On one side the tumour microenvironment can be imaged, such as tumour cell proliferation using 3'-deoxy-3'-^{18}-F-fluorothymidine (^{18}FLT) or tumour cell hypoxia using ^{18}-F-fluoromisonidazole (^{18}FMISO) but also signal transduction pathways can been visualised. Radionuclide labelled monoclonal antibodies directed against the EGFR might in the future be used to select patients for EGFR-targeted therapies, although more clinical trials are necessary to further explore this [51,52]. Although these techniques have the great advantage that a tumour can be monitored during treatment, they cannot distinguish in which cellular (sub-) compartment proteins are being expressed neither can co-expression of different markers be investigated. Possibly, early tumour characterisation by immunohistochemistry supplemented with PET/CT-imaging during treatment can provide an important tool for adaptation and optimisation of treatment plans.

Final remark

The results of this thesis make clear that the tumour microenvironment is of major influence on the response of tumours to treatment. Tumour progression is complex and difficult to understand. The more factors we unravel that are involved in this process the more new questions are raised. The tumour microenvironment is involved in activation of EGFR-driven signalling pathways as well as EGFR-independent activation of proteins such as AKT. The exact mechanisms and elements of the microenvironment that drive EGFR toward PI3-K/AKT signalling remain unclear. The work presented in this thesis describes only details of a complex picture in which EGFR and pAKT are involved not only in a cancer-type specific manner, but also in a patient-tumour-specific way. It highlights the complex interaction and variation of tumours of the same clinical location or stage and necessity for individualised treatment regimes. With the ability to select patients based on tumour characteristics such as hypoxia, we are now able to treat only those patients likely to benefit from hypoxia modifying treatment modalities. Identification and targeting of key molecules of signal transduction pathways in addition to modulation of the tumour microenvironment could further improve the possibility of individualised treatment regimes for the best quality of care while minimizing toxicity levels.

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Chapter 8

Summary

Summary

The tumour microenvironment plays a key role in the treatment response of solid tumours. The central aim of this thesis was to investigate the activation of the EGFR-PI3-K/AKT pathway in biopsies of patients with head and neck cancer and its implication for radiation resistance. Besides the potential prognostic or predictive value, we evaluated expression of key molecules of this pathway in relation to microenvironmental factors, such as vasculature and hypoxia.

In **chapter 2** we discussed the interaction of the EGFR-PI3-K/AKT pathway with the tumour microenvironment and its implications for radiation treatment. The current status of knowledge is reviewed and suggestions for future research are given. Treatment failure through radioresistance of various tumour types is associated with activation of the epidermal growth factor receptor (EGFR). Tumour cell proliferation, DNA-repair, hypoxia, and metastases formation are mechanisms in which EGFR signalling is assumed to have an important role. In clinical trials, a correlation has been demonstrated between high EGFR expression in tumours and poor outcome after radiotherapy. Inhibition of EGFR signalling pathways improves the effectiveness of radiotherapy in solid tumours, like head and neck cancers, by overcoming these main mechanisms of radioresistance. The fact that only a minority of the patients respond to EGFR inhibitors reflects the complexity of interactions between EGFR-dependent signalling pathways and the tumour microenvironment.

In **chapter 3** the purpose was to investigate if EGFR expression levels are associated with response to ARCON in patients with laryngeal carcinoma. Accelerated radiotherapy (AR) improves the poor outcome associated with EGFR overexpression in head and neck cancers. Combining AR with carbogen and nicotinamide (ARCON) counteracts enhanced tumour cell proliferation- and hypoxia-related radioresistance. Paraffin-embedded biopsies of 272 patients randomized between AR and ARCON were processed for immunohistochemical staining of EGFR. A large variation was observed in EGFR fractions between tumours with expression levels ranging from 0-0.93 (median fraction 0.4). No difference in 5-year locoregional control was found between low and high EGFR expressing tumours in the AR arm (69% versus 75%), which is in line with the established effect of AR in EGFR overexpressing tumours. There was, however, a significant association in the ARCON arm: patients with low EGFR levels had a better 5-year locoregional control (88% versus 72% p=0.02) and disease-specific survival (92% versus 77% p=0.01). ARCON improved locoregional control relative to AR only in patients with low EGFR expression (HR 0.34 p=0.009). It is to conclude that EGFR is a predictive biomarker for the selection of patients that will or will not respond to ARCON.

In **chapter 4** the contribution of activated p(hosphorylated)EGFR and its downstream signalling via PI3-K/AKT was investigated. pEGFR, pAKT, hypoxia (by means of pimonidazole staining), and vessels were visualised. It was hypothesised that hypoxic tumour cells with an activated EGFR-PI3-K/AKT pathway could have a survival advantage after treatment with radiotherapy. Both pEGFR (median 0.6%, range 0-34%) and pAKT (median 1.8%, range 0-16%) expression differed between tumours. A significant correlation between pEGFR and pAKT (r_s 0.44, p=0.004) was seen, however, image analysis revealed that this was not always based on spatial coexpression. The correlation between expression of pEGFR and pAKT is indicative of activation of the PI3-K/AKT pathway through phosphorylation of EGFR. Since not all tumours show coexpression to the same extent, other membrane receptors must be involved in the activation of this pathway as well. Un expected and somewhat counterintuitive finding was that low pAKT expression was associated with increased risk of regional recurrence (p<0.05) and distant metastasis (p=0.04). This phenomenon is further investigated in chapter 6.

Chapter 5 focused on the expression of markers involved in epithelial-mesenchymal transition which is required for metastasis formation; E-cadherin and vimentin. E-cadherin is a transmembrane glycoprotein involved in cell-cell adhesion while vimentin is highly expressed in mesenchymal cells. Vimentin is positively correlated with increased metastasis. In this chapter tumour biopsies of 26 head and neck cancer patients were immunohistochemically stained for E-cadherin and vimentin. We found a large variation in E-cadherin (median 17%, range 0-51%) and vimentin (median 0%, range 0-20%) expression between tumours. Tumours with low E-cadherin expression showed a significantly higher incidence of metastasis formation compared to tumours with high expression (81% versus 19%, p=0.004). Enhanced expression of vimentin was associated with a trend towards a higher metastatic risk (33% versus 77%) compared to tumours without expression of vimentin. All patients with low E-cadherin and high vimentin expression (an EMT-phenotype) developed distant metastasis versus only 44% of the other patients (p=0.008). These data indicate that loss of E-cadherin and gain of vimentin may be associated with enhanced migration of tumour cells, leading to higher metastatic risk of head and neck cancer patients.

Chapter 6 aggregated the EGFR-PI3-K/AKT pathway with E-cadherin and vimentin expression data. Targeting AKT is considered as a treatment option for several solid tumour types. However, in chapter 4 we showed that low pAKT expression in the primary tumour was associated with lymph node metastasis. Our aim in chapter 6 was to validate this finding in an independent cohort of laryngeal cancer patients and to examine the effect of pAKT inhibition on EMT of laryngeal cancer cells. We visualised EGFR, pAKT, vimentin, E-cadherin, hypoxia, and blood

vessels in biopsy material of 78 patients. In six laryngeal cancer cell lines E-cadherin and vimentin mRNA and protein expression was quantified by respectively real-time polymerase chain reaction (q-pcr) and by immunohistochemistry before and after treatment with the pAKT inhibitor MK-2206. We found a significant correlation between low pAKT in the primary tumour and positive lymph node status (p=0.0005). Tumours with lymph node metastases had approximately 10-fold lower median pAKT values compared to tumours without lymph node metastases, validating our previous findings. After inhibition of pAKT in laryngeal cancer cells with MK-2206 upregulation of vimentin and a downregulation of E-cadherin occurred, consistent with EMT induction predisposing for an increased metastatic risk. These results describe a complex picture in which pAKT inhibition should be considered in a cancer-type specific manner. We recommend that new pAKT inhibitors should be tested for potential stimulation of EMT and be introduced in the clinic prudently.

Finally, **chapter 7** provides a general discussion of findings presented in this thesis and future considerations are highlighted.

Chapter 9

Samenvatting

Samenvatting

Het doel van dit proefschrift was om de functie van de EGFR-PI3-K/AKT signaleringsroute te onderzoeken in biopten van patiënten met hoofd-halstumoren en te testen welke rol deze speelt bij resistentie tegen radiotherapie. Naast het bepalen van de mogelijke prognostische en predictieve (voorspellende) waarde van EGFR in deze patiënten werd ook de expressie van EGFR en pAKT in te tumor gerelateerd aan factoren binnen het tumor-micromilieu, zoals hypoxie (zuurstofgebrek) en de verdeling van de bloedvaten.

Hoofdstuk 1 geeft een introductie over hoofd-halstumoren, radioresistentie en de EGFR-PI3-K/AKT signaleringsroute. Onderstaande is een korte samenvatting van dit hoofdstuk.

Hoofd-halstumoren

Hoofd-halskanker behoort tot de tien meest voorkomende tumor-soorten met een incidentie van ongeveer 2400 patiënten per jaar in Nederland. Hoofd-halstumoren zijn meestal afkomstig van het slijmvlies van de larynx, farynx en neus- en mondholte. De belangrijkste oorzaken van het krijgen van hoofd-halstumoren zijn roken en overmatig alcoholgebruik. Een infectie met het humaan papillomavirus (HPV) is in toenemende mate verantwoordelijk voor het ontstaan van orofarynx-tumoren bij relatief jonge, niet rokende patiënten. De initiële klachten zijn meestal niet-specifiek zoals keelpijn en daardoor presenteren de meeste patiënten zich met vrij grote tumoren die vaak al metastasen (uitzaaiingen) in de lymfeklieren van de hals vertonen. Doordat chirurgie in het hoofd-halsgebied vaak tot belangrijk functieverlies leidt is de voorkeursbehandeling voor deze tumoren radiotherapie; deze therapie is effectief in een vroeg stadium van de ziekte maar minder in vergevorderd stadia en slechts palliatief (niet gericht op genezing) bij tumoren met metastasen op afstand. In de afgelopen jaren is er veel onderzoek gedaan naar het optimaliseren van behandelingsmethoden om de kans op overleving te vergroten. Voorbeelden hiervan zijn het versnellen (accelereren) van radiotherapie schema's of het combineren van radiotherapie met chemotherapie of medicijnen die ingrijpen op moleculaire doelen in de tumorcel. Een beperkt deel van de patiënten (ongeveer 15%) heeft voordeel van deze behandelingen maar helaas ondervindt iedereen de bijwerkingen ervan. Het is dus noodzakelijk om patiënten voor de juiste behandeling beter te kunnen selecteren.

Mechanismen betrokken bij radioresistentie

Het tumor-micromilieu speelt een belangrijke rol bij de uitkomst van radiotherapie. Hoe goed tumorcellen reageren op de behandeling hangt af van de tumoreigen gevoeligheid voor bestraling (intrinsieke radiosensitiviteit), de snelheid

van deling (proliferatie), zuurstofgebrek (hypoxie) en de mogelijkheid tot metastasering. Intrinsieke radiosensitiviteit wordt bepaald door de mate waarin tumorcellen de bestralingsschade aan het DNA kunnen herstellen. Versnelde proliferatie treedt op doordat tumorcellen reageren door naarmate de behandeling vordert actiever te gaan delen. Dit kan tegengegaan worden door radiotherapie in een kortere periode, geaccelereerd, te geven. Hypoxie komt voor in bijna alle hoofd-halstumoren. Acute hypoxie ontstaat door een willekeurige en kortdurende sluiting van de slecht gevormde tumor bloedvaten terwijl chronische hypoxie het gevolg is van een te grote afstand van de tumorcel naar het dichtstbijzijnde bloedvat. Zoals te zien is in figuur 1 van hoofdstuk 1 kunnen beide vormen van hypoxie in dezelfde tumor voorkomen en is het gevolg dat er een tekort aan zuurstof en voedingsstoffen in de tumorcel optreedt. Zuurstof is essentieel voor het effect van bestraling omdat het fungeert als intermediair bij het ontstaan van DNA schade. Indirect kan hypoxie leiden tot activatie van genen en eiwitten die verantwoordelijk zijn voor tumor progressie of metastasering en uiteindelijk tot een slechtere prognose van de patiënt. Bij metastasering ondergaan tumorcellen veranderingen waarbij ze los komen van de primaire tumor, kunnen gaan zwerven in de bloed- of lymfebaan en opnieuw kunnen gaan hechten op een tweede lokalisatie in het lichaam. Voor die eerste stap is een proces genaamd epitheliale-mesenchymale transitie (EMT) noodzakelijk. Hierbij veranderen de tumorcellen van een hechtende, epitheliale cel in een mesenchymale cel. Metastasen hebben een zeer grote impact op de prognose van de patiënt en daarom is het erg belangrijk om deze mechanismen te begrijpen.

EGFR-PI3-K/AKT signaleringsroute

EGFR is een eiwit dat zich op het membraan van de tumorcel bevindt en in hoofd-hals-kanker in grote mate tot expressie komt. Zoals figuur 2 in hoofdstuk 1 laat zien, kan activatie van EGFR leiden tot activatie van verschillende signaleringsroutes in de tumorcel, waaronder de PI3-K/AKT route. Deze signaleringsroute is belangrijk voor de respons op radiotherapie doordat het een groot aantal cellulaire functies reguleert, waaronder de mogelijkheid tot proliferatie, DNA-herstel en metastasering. Uit eerder onderzoek is al gebleken dat EGFR een voorspellende waarde heeft in solide tumoren, waarbij er een sterke correlatie is aangetoond tussen een hoge EGFR expressie in de tumor en een slechtere prognose voor de patiënt. Een klinische studie heeft laten zien dat toevoeging van een therapie gericht op het remmen van EGFR signalering (met behulp van het geneesmiddel cetuximab) aan radiotherapie leidt tot een verbeterde locoregionale controle bij patiënten met hoofd-halstumoren vergeleken met de patiënten die alleen bestraald werden. Helaas blijkt die niet voor alle patiënten te werken, terwijl zij wel allemaal aan deze intensievere behandeling en bijwerkingen worden blootgesteld. Het is in de laatste jaren duidelijk geworden dat het tumor-micromilieu een grote rol speelt in EGFR signalering en activatie van de PI3-K/AKT route. In dezelfde tumor zijn er tumorcellen met actief EGFR terwijl ze

geen actief AKT bezitten, maar ook delen waren AKT actief is zonder dat er EGFR aanwezig is. Hieruit blijkt dat het erop lijkt dat de activatie van AKT niet altijd door EGFR plaatsvindt maar er ook andere factoren een rol spelen. Dit geeft aan dat de interactie tussen het tumor-micromilieu en EGFR signaleringsroutes complex is.

In **hoofdstuk 2** wordt de interactie tussen de tumor en zijn micromilieu besproken en wat dit voor gevolgen heeft voor radiotherapie. Er wordt een overzicht gegeven van de huidige stand van zaken en aanbevelingen gegeven voor toekomstig onderzoek. Het falen van een behandeling door ongevoeligheid van tumoren voor bestraling is geassocieerd met activatie van EGFR. Tumorcelproliferatie, herstellen van DNA-schade, hypoxie en metastasering zijn vier mechanismen waarbij signaleringsroutes via EGFR een belangrijke rol in spelen. In klinische trials is een correlatie aangetoond tussen een hoge expressie van EGFR in tumoren en een slechte overleving van de patiënt na radiotherapie. Het blokkeren van deze EGFR signaleringsroutes in de tumor verbetert de effectiviteit van radiotherapie in solide tumoren, waaronder hoofd-halstumoren. Dat de interactie tussen EGFR afhankelijke signaleringsroutes in tumoren en het tumor micromilieu complex is blijkt uit het feit maar een klein deel van de patiënten (maximaal 15%) met een hoge EGFR expressie baat heeft bij behandeling met deze EGFR-remmers.

Het doel van **hoofdstuk 3** was te onderzoeken of expressie van EGFR geassocieerd is met de respons van patiënten met larynxtumoren op behandeling met ARCON: geaccelereerde radiotherapie (AR) met carbogeen en nicotinamide (CON). AR verbetert de slechte prognose die gerelateerd is aan overexpressie van EGFR in hoofd-halstumoren. Door het geven van ARCON wordt zowel versnelde tumorcelproliferatie als hypoxie-gerelateerde radioresistentie tegengegaan. Paraffine coupes van 272 patiënten die gerandomiseerd waren tussen behandeling met AR of ARCON werden immunohistochemisch gekleurd voor EGFR. Door middel van analyses met automatische beeldverwerking werden fracties EGFR bepaald: de hoeveelheid EGFR positieve tumorcellen gedeeld door het totaal aantal tumorcellen. Een grote variatie werd gevonden tussen de verschillende tumoren met EGFR fracties tussen 0 en 0,93 (mediane fractie 0,4). Er was geen significant verschil tussen hoge en lage EGFR expressie in de 5-jaars locoregionale controle in de patiënten behandeld met AR alleen (69% versus 75%). Dit komt overeen met eerdere studies die aantonen dat het accelereren van radiotherapie schema's de prognose van tumoren met hoge EGFR expressie verbeteren tot dezelfde prognose van tumoren met lage EGFR expressie. Wel was er een significante correlatie in de patiënten die behandeld waren met ARCON: patiënten met een lage EGFR fractie hadden een betere 5-jaars locoregionale controle dan degenen met een hoge EGFR fractie (88% versus 72%, p=0,02) en ook ziektevrije overleving (92% versus 77%, p=0,01). Behandeling met ARCON verbeterde

de locoregionale controle ten opzichte van behandeling met AR alleen voor patiënten met een lage EGFR expressie (HR 0,34 p=0,009). Uit dit onderzoek blijkt dat EGFR expressie een predictieve biomarker is om te voorspellen of patiënten wel of geen baat zullen hebben bij behandeling met ARCON.

In **hoofdstuk 4** wordt de geactiveerde vorm van EGFR, gefosforyleerd (p)EGFR, en de signaleringsroute PI3-K/AKT nader onderzocht. Hypoxische tumoren die de EGFR-PI3-K/AKT signaleringsroute kunnen activeren hebben mogelijk een overlevingsvoordeel na behandeling met radiotherapie. Ingevroren biopten van verschillende hoofd-halstumoren werden gekleurd middels immunofluorescentie voor pEGFR, pAKT, hypoxie en bloedvaten, en fracties werden bepaald. Zowel pEGFR (mediaan 0,6%, min-max 0-34%) en pAKT (mediaan 1,8%, min-max 0-16%) expressie varieerde sterk tussen tumoren. Er was een significante correlatie tussen pEGFR en pAKT (r_s 0.44, p=0.004), maar dit bleek niet altijd gebaseerd op activatie van beide eiwitten in dezelfde tumorcel. Een lage expressie van pAKT was geassocieerd met lymfeklier metastasen op het moment van diagnose (0.5% resp. 5.8% p=0.001). Lage pAKT expressie lijkt ook te leiden tot een verhoogd risico op zowel regionale terugkeer van de ziekte (p<0.05) als metastasen op afstand (p=0.04). De correlatie tussen pEGFR en pAKT expressie in tumoren zou erop kunnen duiden dat activatie van de PI3-K/AKT signaleringsroute door middel van activatie van EGFR tot stand komt. Echter, aangezien niet altijd pEGFR en pAKT in dezelfde tumorcel tot expressie komt moeten er andere factoren, bijvoorbeeld andere signaleringsroutes of mutaties in de tumor, een rol spelen bij activering van AKT.

Hoofdstuk 5 richt zich op de expressie van de markers E-cadherine en vimentine die een rol spelen bij epitheliale-mesenchymale transitie (EMT), een proces dat nodig is voor tumorcellen om te kunnen metastaseren. E-cadherine is een eiwit dat zich op het membraan van een cel bevindt en een rol speelt bij cel-cel contact en aanhechting. Vimentine komt in mesenchymale cellen tot expressie en correleert in eerdere onderzoeken met verhoogde kans op metastasering van tumoren. Voor dit onderzoek werden biopten van 26 patiënten gekleurd voor E-cadherine en vimentine en werden fracties bepaald. Er bestond een grote variatie tussen de tumoren voor zowel de expressie van E-cadherine (mediaan 17%, min-max 0-51%) als van vimentine (mediaan 0%, min-max 0-20%). Patiënten met tumoren met een lage fractie E-cadherine hadden significant meer metastasen vergeleken met tumoren die een hoge E-cadherine fractie hadden (81% versus 19%, p=0.004). Een verhoogde expressie van vimentine was geassocieerd met een trend op een hoger risico op metastasen (33% versus 77%). Alle patiënten met zowel een lage E-cadherine als een hoge vimentine expressie in de tumor (zoals gezien bij het proces van EMT) kregen afstandsmetastasen vergeleken met slechts 44% van de overige patiënten. Deze data tonen aan dat het verlies van E-cadherine gecombineerd met een verhoogde expressie

van vimentine in tumoren geassocieerd is met een versterkte migratie van tumorcellen en uiteindelijk tot een verhoogd risico op de vorming van metastasen bij hoofd-hals kanker kan leiden.

In **hoofdstuk 6** worden de twee voorgaande hoofdstukken samengebracht; de EGFR-PI3-K/AKT signaleringsroute en de EMT-markers E-cadherine en vimentine. Therapieën die als doel hebben om AKT te verminderen en hierdoor de prognose van de patiënt te verbeteren zijn tegenwoordig een behandelingsoptie voor verschillende solide tumoren. In hoofdstuk 4 van dit proefschrift werd aangetoond dat een lage pAKT expressie in de tumor geassocieerd is met lymfekliermetastasen In dit hoofdstuk is het doel om deze bevinding te valideren in een apart cohort van patiënten met uitsluitend larynxtumoren. Tevens werd het effect van pAKT remming op EMT in cellijnen van larynxkanker bestudeerd. In biopten van 78 patiënten werden EGFR, pAKT, E-cadherine, vimentine, hypoxie en bloedvaten gekleurd. Daarnaast werd in 6 cellijnen de mRNA expressie van E-cadherine en vimentine bepaald door middel van een kwantitatieve polymerase kettingreactie (q-pcr) en de expressie van de eiwitten met immunohistochemische kleuringen voor en na behandeling van deze cellen met de pAKT remmer MK-2206. Bevestigd werden onze onverwachte resultaten uit hoofdstuk 4, dat een lage expressie van pAKT in tumoren gecorreleerd is met lymfeklier metastasen (p=0.005). Tumoren met lymfekliermetastasen hadden ongeveer een 10 maal lagere hoeveelheid pAKT vergeleken met tumoren zonder lymfekliermetastasen. Na remming van pAKT in larynxkankercelllijnen met MK-2206 werd een verlaging gezien van E-cadherine expressie en een verhoogde vimentine expressie, consistent met inductie van EMT en daardoor duidend op een verhoogd risico op metastasevorming. Op basis van deze bevindingen wordt aanbevolen dat, voor introductie in de kliniek, nieuwe pAKT remmers getest zouden moeten worden op mogelijke stimulatie van EMT in hoofd-halskanker.

Tenslotte wordt in **hoofdstuk 7** de resultaten van alle hoofdstukken in een breder perspectief besproken en worden er suggesties voor toekomstig onderzoek gegeven. De resultaten van dit proefschrift maken duidelijk dat het tumor micromilieu een grote invloed heeft op de respons van tumoren op de gekozen behandeling. De progressie van tumoren is een complex en moeilijk te doorgronden proces. Bij elke nieuwe ontdekking komen nieuwe vragen naar boven. Het tumor micromilieu is zowel betrokken bij de activatie van EGFR gestuurde signaleringsroutes als bij de activatie van EGFR onafhankelijke eiwitten zoals AKT. Welke mechanismes en elementen van dit tumor micromilieu leiden tot activatie van de EGFR-PI3-K/AKT signaleringsroute blijft echter onduidelijk. Het werk beschreven in dit proefschrift zijn slechts details van een complex geheel waarbij EGFR en pAKT betrokken zijn, zowel in een tumor-type (namelijk hoofd-halskanker) als patiënt-tumor specifieke manier. De variatie

tussen tumoren die tot dezelfde klinische locatie of stadium behoren maken duidelijk dat het noodzakelijk is patiënten geïndividualiseerd te kunnen behandelen. Met de mogelijkheid om patiënten te selecteren op basis van tumor specifieke kenmerken, zoals hypoxie, zijn we in staat om alleen die patiënten te behandelen die baat hebben bij zogenoemde hypoxie modificerende therapieën. De identificatie van belangrijke signaleringsroutes kan leiden tot de ontwikkeling van therapieën die specifiek op gericht zijn op tumor specifieke eigenschappen. Het combineren van deze verschillende therapieën kan bijdragen aan de mogelijkheid tot geïndividualiseerde behandelingsstrategieën zodat het mogelijk wordt om te kiezen voor de meest geschikte behandeling met de laagste toxiciteit en de beste kwaliteit van leven.

Dankwoord

Curriculum Vitae

List of publications

Dankwoord

Het laatste hoofdstuk…het enige hoofdstuk in mijn proefschrift wat niet geschreven, gecorrigeerd, geschrapt, herschreven, gereviseerd en becommentarieerd is. En dat terwijl dit waarschijnlijk het meest gelezen hoofdstuk zal zijn. Ik wil iedereen bedanken die de afgelopen jaren mij geholpen heeft dit proefschrift voor elkaar te krijgen; iedereen die inhoudelijke input gegeven heeft, maar ook zeker diegene die een voor luisterend oor zorgden en die er waren om te sporten en ontspannen. Zonder deze combinatie had ik het nooit volbracht!

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Hora Est

Curriculum Vitae

Monique Maria Nijkamp werd geboren op 6 augustus 1981 te Harderwijk. Zij groeide op in Harderwijk en Dronten en kwam uiteindelijk in het Twents Losser terecht. Van 1993 tot 1998 volgde zij de HAVO aan het Twents Carmellyceum te Oldenzaal. Na een kort uitstapje op de pabo begon zij in 1999 met de opleiding Hoger Laboratorium Onderwijs aan de Saxion Hogescholen te Enschede met Medische Microbiologie als afstudeerrichting. Na het behalen van haar diploma vervolgde zij haar weg in Nijmegen waar aan de Radboud Universiteit de studie Biomedische Wetenschappen werd gevolgd. Met een masterstage Toxicologie bij het Rijksinstituut voor Volksgezondheid en Milieu en een maatschappelijk profiel stage bij de afdeling medische milieukunde van de (toenmalige) Hulpverleningsdienst Gelderland Midden werd deze studie afgerond. Na twee jaar werkzaam geweest te zijn als milieugezondheidskundige bij Bureau Gezondheid, Milieu en Veiligheid van de GGD'en Brabant/Zeeland besloot Monique de kans om te promoveren aan te grijpen. In 2009 werd bij de afdeling Radiotherapie begonnen met het onderzoek waarvan de resultaten staan beschreven in dit proefschrift. Gedurende deze periode heeft zij twee maal de Klaas Breur Fonds reisbeurs gewonnen en op meerdere congressen haar onderzoek mogen presenteren.
Op dit moment werkt Monique bij de afdeling Consumenten en Productveiligheid van het RIVM waar zij zich bezighoudt met blootstellingschatting/beoordeling van chemische stoffen in consumentenproducten.

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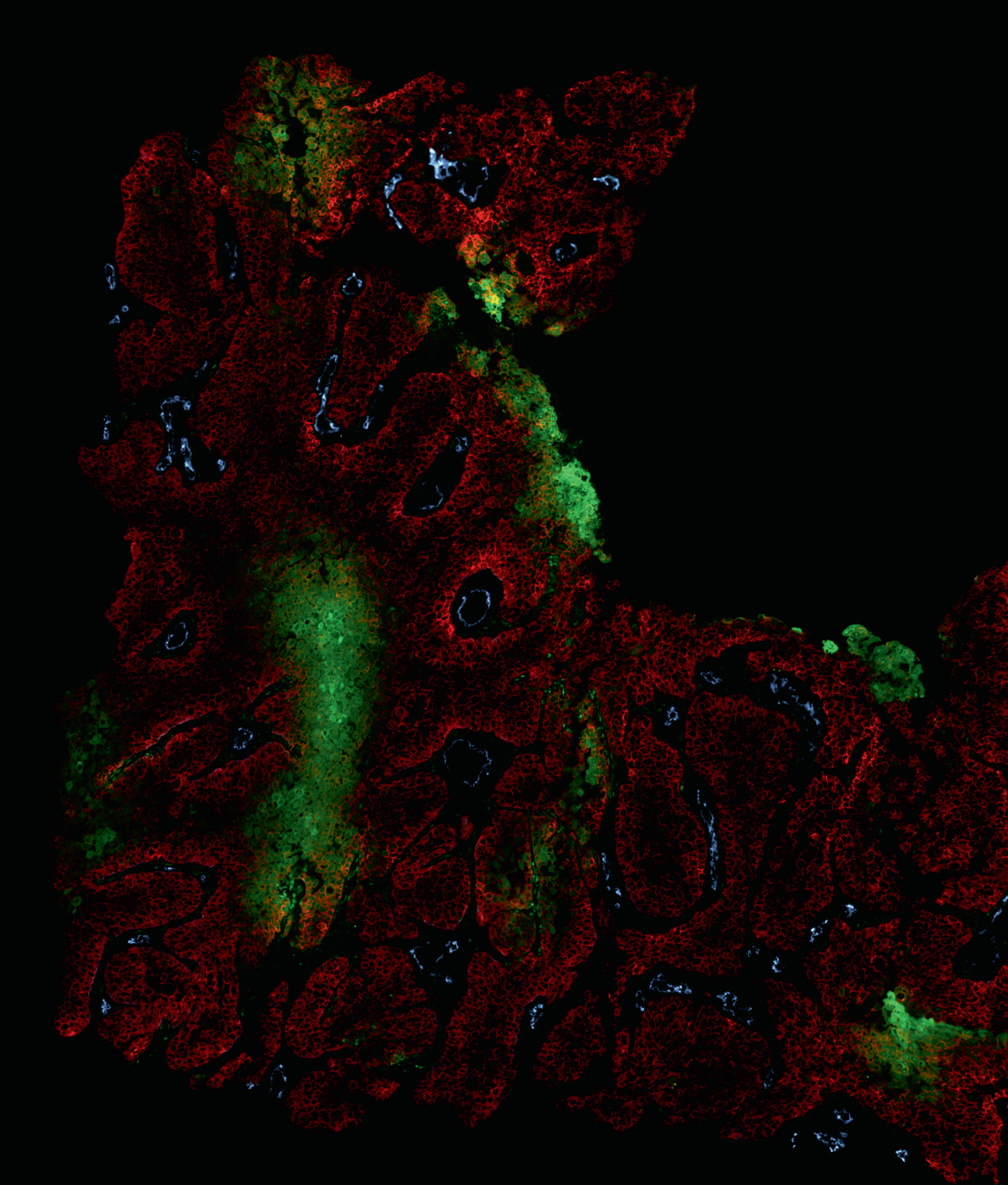

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