Oxygen-enhanced radiotherapy in advanced laryngeal cancer

An analysis of the predictive value of hypoxia-related markers

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Contents

List of abbreviations

AR	Accelerated Radiotherapy
ARCON	Accelerated Radiotherapy combined with Carbogen breathing and Nicotinamide
ATP	Adenosinetrifosfaat
CAIX	carbonic anhydrase IX
CT	computed tomography
DAB	diaminobenzidine
DFS	disease-free survival
EF-5	2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)acetamide
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
GLUT-1/-3	glucose transporter-1/-3
Gy	Gray [Jxkg^{-1}]
h	hour
Hb	hemoglobin
HE	haematoxylin-eosin staining
HIF-1	hypoxia-inducible factor-1
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
IdUrd	iododeoxyuridine
Ki-67	endogenous proliferation marker
LC	local control
LDH-5	lactatedehydrogenase-5
LI	labeling index
MCT1/4	monocarboxylate transporter 1/4
MFS	metastasis-free survival
MRI	magnetic resonance imaging
M-stage	distant metastasis
N-stage	regional lymph node metastasis
OS	overall survival
PAD	primary antibody diluent
PBS	phosphate buffered saline
PI3-K	phosphatidyl-inositol-3' kinase
pimonidazole	1-([2-hydroxy-3-piperidinyl)propyl]-2-nitroimidazole hydrochloride
pO_2	oxygen partial pressure
RC	regional control
RVA	relative vascular area
TNM	classification of malignant tumors
TPZ	tirapazamine
T-stage	extent of primary tumor
VD	vascular density
VEGF	vascular endothelial growth factor
VHL	Von Hippel-Lindau
WHO	World Health Organization

1

General introduction and outline

Based on Molecular oncology 2008; 2 (1): 41-53 Review

Laryngeal carcinoma

Every year around 700 cases of laryngeal cancer are diagnosed in the Netherlands.[1] The disease is more prevalent in males than females with a factor 7:1, although incidence is increasing in females.[2] This is probably due to the changing smoking habits in the past decades, smoking being the most important etiological factor for larynx carcinoma. Other etiological factors include alcohol use[3] and to a lesser extent occupational exposures[4] and human papilloma virus (HPV)[5].

Squamous cell carcinoma is by far the most prevalent histopathological diagnosis, other histologies are rare. Two third of the laryngeal cancers is located supraglottic, about one third glottic and subglottic localisation is very rare (about 2% of cases).[6] The anatomy of the larynx illustrating the different subsites is shown in Figure 1.

The treatment for the early stages comprises laser surgery or radiotherapy with excellent long term results.[7] In the more advanced stages, it is still a challenge to achieve long term local control and survival. The last decades a shift in therapy has occurred for these tumours from laryngectomy to radiotherapy to preserve organ function, with a similar patient outcome for both modalities.[8] Still a significant subset of patients is faced in time with locoregional recurrence or distant metastases. Hypoxia (the lack of oxygen) is an important resistance mechanism that can cause therapy failure.

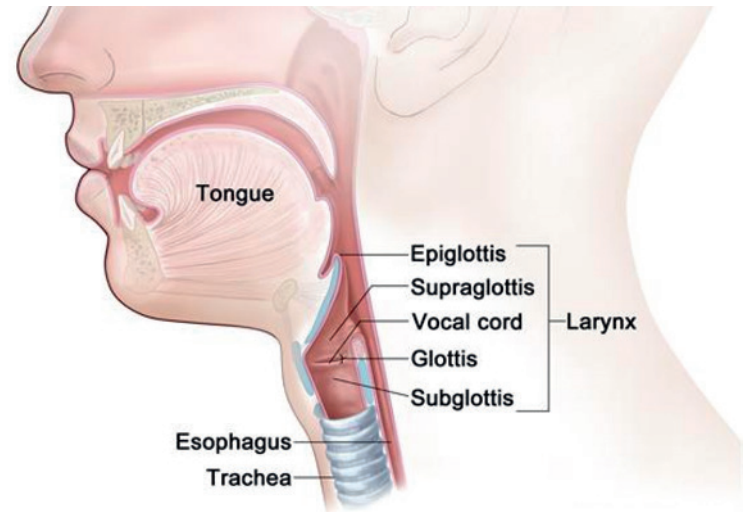

Fig. 1 Anatomy of the larynx.

Radiotherapy and the oxygen effect

The effect of radiotherapy on tumor cells is strongly dependent on the presence or absence of oxygen, the so called oxygen-effect.[9] Under hypoxic circumstances, a higher radiation dose is needed to cause the same biological effect. This relationship is described by the oxygen enhancement ratio (OER) and is defined by the radiation dose in hypoxia divided by the radiation dose in air. The enhancement of radiation damage by oxygen is explained by the fixation of DNA damage by oxygen. Radiation causes highly reactive free radicals in the DNA of the tumor cell, either direct or indirect. These free radicals rapidly react with oxygen which eventually causes a stable chemical fixation of the DNA damage. In the absence of oxygen the free radicals have more time to restore their original form.

Hypoxia and the tumor microenvironment

The tumour microenvironment is of great importance, influencing malignant cells in various ways.[10] Within this microenvironment, hypoxia is an extensively studied parameter, with relevance in almost all types of solid tumours. As early as 1936 Mottram mentions the relative insensitivity of mammalian cells under anaerobic conditions.[11] Gray described in 1953 the presence of hypoxia in murine tumour cells and the associated reduced sensitivity to radiotherapy.[12] Hypoxia has negative implications for clinical outcome. This is probably based on two distinct principles: hypoxic cells are more resistant to radiotherapy and chemotherapy, and they give rise to genetic instability and more aggressive phenotypes.

Especially the increased resistance to radiotherapy is a well-known phenomenon associated with tumour hypoxia, most studied in head and neck cancer and cervical carcinoma.[13,14] Besides this increased resistance to radiotherapy, there is evidence that hypoxic cells are responsible for decreased sensitivity to certain chemotherapeutic agents as well, such as doxorubicin, 5-fluorouracil and methotrexate. These data are mainly derived from animal and in vitro studies.[15-17] There is scarce recent literature available on this subject.

Besides this resistance to chemo- and radiotherapy, surgically treated soft-tissue sarcomas and cervical carcinomas also exhibit hypoxia as a prognostic factor for poor survival.[13,18] A correlation was found between the rate of distant metastases and tumour oxygenation in soft tissue sarcoma.[19] Furthermore, in cervical cancer it has been proven that hypoxic tumours exhibit more frequent parametrial spread and lymph-vascular space involvement.[13] A subgroup of hypoxic tumours with diminished apoptotic potential showed increased lymphatic spread and higher probability of recurrence as well.[20] These findings indicate that hypoxia stimulates tumour cells to develop towards a more invasive phenotype and can be regarded as a sign of tumour aggressiveness.

Pathophysiology of hypoxia

In most malignant tumours there is an imbalance between the supply and consumption of oxygen, leading to hypoxic and even anoxic regions. During the rapid growth of a tumour an aberrant, chaotic microvasculature develops. Tumours exhibit a vascular network with a wide range of vessel diameters, intervascular distances and interbranching distances unlike those seen in normal tissue.[21] The morphological and functional deformed blood vessels diminish the oxygen delivery to the tumour cells, resulting in a low oxygen microenvironment. Another factor contributing to a decrease in oxygen supply is tumour-associated and therapy-induced anaemia, leading to a diminished oxygen transport capacity of the blood (anaemic hypoxia).[22]

From a pathophysiological point of view hypoxia can be divided into acute, or perfusion-limited hypoxia and chronic, or diffusion-limited hypoxia, although this division is arbitrary.[23] Acute hypoxia is often transient and is caused by a temporary disruption in blood flow as a result of an occlusion or rise in interstitial fluid pressure. In xenografts only a small part of the tumour hypoxia is of the intermittent type.[24]

Chronic hypoxia arises with an increased distance of tumour cells from the microvessels (> 70 µm) in the distorted architecture of the vascular network. Acute and chronic hypoxia combine in a heterogeneous pattern throughout the tumour so that severe, intermediate and low levels of hypoxia can be identified. From a clinical standpoint it has been proposed that the intermediate and transient hypoxic cells are the most important for prognosis.[25] These cells are (temporarily) resistant to therapy and still have the ability to proliferate.[26]

Because the degree of hypoxia is extremely variable on a continuous scale, a strict cut-off value to discriminate normoxic from hypoxic cells does not exist. Critical metabolic processes fail at different pO_2-levels (Figure 2). A median O_2 partial pressure of less than 10 mm Hg already results in ATP depletion. The oxidative phosphorylation gradually declines below this value till it ceases at O_2 partial pressures of less than 0.5 mm Hg. Radioresistance may already occur below 25-30 mm Hg.[27] In most experiments, values from 0.5 mm Hg to 10 mm Hg have been used as a cut-off value to discriminate normoxic from hypoxic tumour cells.

Tumor metabolism

Malignant tumors often exhibit an altered metabolism compared to normal tissues. This phenomenon can be explained by several underlying mechanisms. First of all, the genetic changes related to a high proliferation rate, as observed in many tumors, lead to an increased metabolism.[28] Another important reason for a changed metabolism is the adaptation of tumor cells to the microenvironment. Under circumstances of severe hypoxia, cells are forced to use anaerobic glycolysis as their primary energy source, the Pasteur effect (Figure 2).[29] Normal cells convert to oxidative phosphorylation when oxygen levels are restored. In contrast, tumor cells can use glycolysis even in the presence of

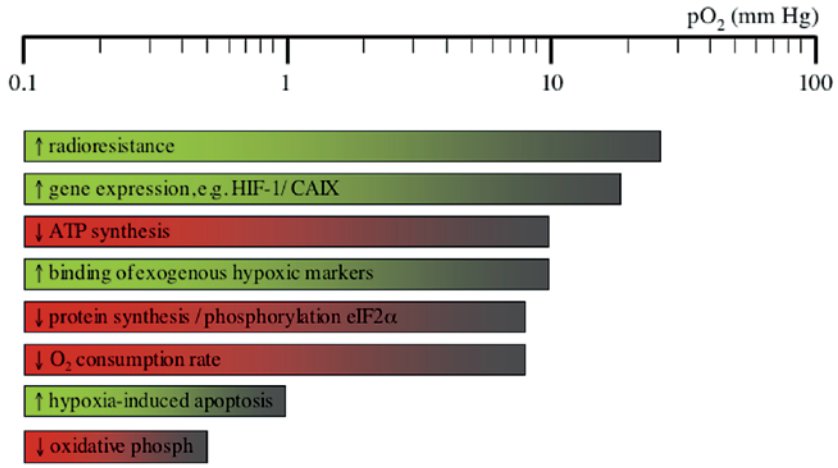

Fig. 2 Cellular adaptation to hypoxia. The bars show the approximate hypoxic values below which cellular responses gradually change.

sufficient amounts of oxygen. This is called the Warburg effect, a manifestation of a modification of the tumor cell metabolism.[30] Due to a high level of aerobic glycolysis, in many tumor cells, glucose consumption is substantially higher than in normal cells.[31,32]

The consequence of the high rate of glycolysis in malignant cells is the production of large amounts of lactic acid. An interesting observation made by Sonveaux et al. is the preference of tumor cells for lactic acid over glucose as the primary energy source.[33] This creates the perfect conditions for a symbiosis between anaerobic glycolytic cells and aerobic tumor cells[33] or aerobic stromal cells, as described in colorectal carcinomas.[34]

Hypoxia and proliferation

In many types of cancer the pathways that regulate cell proliferation are altered and lead to the deregulated growth characteristic of malignant tumours. Proliferation is a well-known adverse prognostic factor in all types of cancer. It is correlated with differentiation grade, tumor size, nodal stage and DNA aneuploidy in various tumor sites and with hormone receptor status in breast cancer.[35] In head and neck cancer high proliferation index is correlated with tumor characteristics and poor outcome in numerous studies.[36] This emphasizes the relationship between proliferation and tumour aggressiveness.

There are several methods to determine the proliferation rate of tumours, that can measure different parameters like the growth fraction, a specific phase of the cell cycle or the cell cycle time.[36] A widely used immunohistochemical proliferation marker that is also used in clinical practice is Ki-67. This endogenous marker is expressed in all phases of the cell cycle, with exception of the G0 phase. Ki-67 has been correlated to clinical outcome in

numerous studies, with the main focus on breast cancer.[37] Several studies assessed the prognostic value of Ki-67 in laryngeal cancer specifically, but most in surgically treated patients. Some of those have shown a correlation of Ki-67 labeling index with recurrence or disease-free survival, but none of them have established a correlation with overall survival.[36]

Although a direct relationship between hypoxia and proliferation has never been found, in theory there is a mutual relationship between proliferation and hypoxia. Highly proliferating tumours with rapid growth will easily give rise to hypoxic areas. Furthermore, hypoxic conditions can drive a cell towards a more aggressive phenotype with higher proliferation rate and more regional and distant metastasis.[38]

Hypoxic cells that have retained their proliferative capacity could be the cause of persistent or recurrent disease. A previous study showed the presence of such a subgroup and its correlation with disease-free survival.[26]

Measuring hypoxia

Over the years different methods have been developed for assessing the level of hypoxia in tumours in vivo, with the polarographic oxygen electrode mostly used as the gold standard. However, the invasive nature of this method led to the development of other techniques. Nowadays, the focus has shifted to exogenous markers, like EF5 and pimonidazole and a wide range of hypoxia-related endogenous markers (Figure 3). The cellular responses to hypoxia are complex, with many different genes and proteins involved. Measuring hypoxia with imaging modalities such as magnetic resonance imaging (MRI) and positron emission tomography (PET) is another promising field of research, which is a subject beyond this thesis.

Polarographic needle electrode

Being regarded as the gold standard for a long time, the polarographic oxygen electrode has been extensively used for quantifying hypoxia in both animal studies and human tumours.[39,40] With this invasive technique oxygen concentrations can be measured directly at many different positions in the tumour and within a short time frame. Although the Eppendorf electrode, introduced in the late 1980's, is a vast improvement compared to the older systems, the method still has some major drawbacks. It is limited to accessible tumour sites like head and neck and cervical cancer, disrupts the tissue and it has a large inter-observer variability.[41] Another restriction of this technique is the failure to distinguish necrotic areas from viable tumour tissue and to discern the patterns of hypoxia.

Exogenous markers

The limitations of the needle electrode led to the development of exogenous markers, the 2-nitroimidazoles, to measure hypoxia (Figure 3A and B). Two markers are approved for

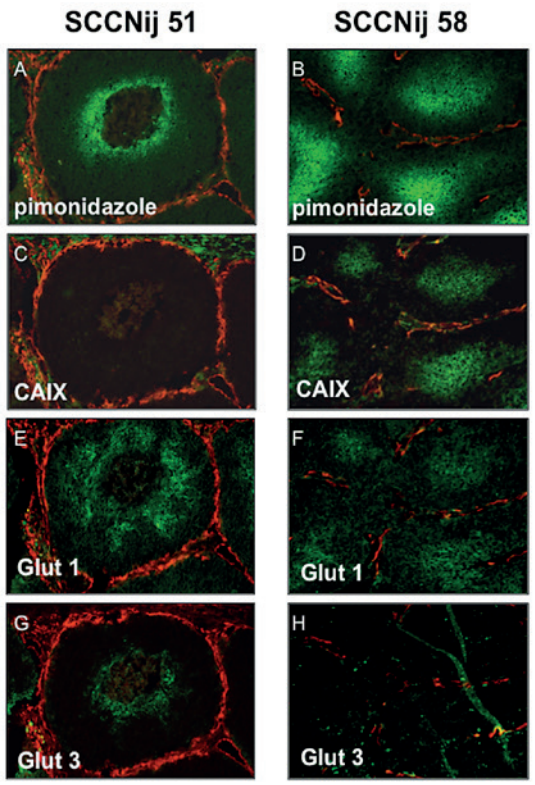

Fig. 3 Photomicrographs of two human squamous-cell carcinoma xenograft cell lines (SCCNij51 and SCCNij58) with immunofluorescent staining. The images show the differences in colocalisation of the exogenous marker pimonidazole (**A** and **B**) and three endogenous hypoxic markers: CAIX (**C** and **D**), Glut-1 (**E** and **F**) and Glut-3 (**G** and **H**) (all in green), relative to the vasculature (in red).

clinical use: pimonidazole, (1-[(2-hydroxy-3-piperidinyl)propyl]-2-nitroimidazole hydrochloride, and EF5, [2-(2-nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide]. These markers can be administered intravenously and are reduced and bound to thiol-containing proteins in viable hypoxic cells. Pimonidazole and EF5 have been proven to be reliable hypoxic markers, with a good correlation with the radiobiologically hypoxic fraction[42,43] and pO_2.[42,44]

Several studies have shown the prognostic value of the 2-nitroimidazoles in a clinical setting. In head and neck cancer a correlation was found between the degree of hypoxia estimated by 2-nitroimidazole binding and the locoregional control and event-free-survival.[45,46] One study performed in patients with cervical carcinoma did not show this

association.[47] In patients with head and neck cancer treated in a phase II trial with ARCON (accelerated radiotherapy with carbogen and nicotinamide), a hypoxia-modifying therapy, pimonidazole demonstrated a predictive value as well.[45]

An oral prescription of pimonidazole has become available, which remains to be validated.[48] Recently, it has been FDA approved. For clinical use it would be a progress, making the administration of pimonidazole more convenient.

Endogenous hypoxia-related markers

An alternative method not relying on injected markers to quantify hypoxia is the use of endogenous markers, an extensive range of potential markers having been studied over the last years.[49] Endogenous markers are proteins upregulated in association with hypoxia and can be measured in blood plasma or immunohistochemically on tumour biopsies, with the advantage that no prior infusion of markers is necessary and therefore archived material can be used. No single marker has consistently demonstrated strong prognostic power in clinical practice as of yet, although a correlation with patient outcome has been found for several markers. Attempts have been made to combine various markers to create a hypoxia-prognostic profile, with moderate success.[50,51] The markers that apply to this thesis will be highlighted, with emphasis on their role in the hypoxic molecular response, the tumor metabolism and their clinical significance.

HIF-1

Hypoxia initiates a complicated response involving a plethora of different molecular pathways. These pathways modulate several cellular functions, like proliferation, apoptosis, angiogenesis, pH balance and anaerobic glycolysis. The HIF-1 pathway with its numerous downstream targets is the key controller in this reaction (Figure 4).

The transcription factor HIF-1 is a heterodimer consisting of two subunits: HIF-1α and HIF-1β. HIF-1β is constitutively active, while HIF-1α is rapidly degraded in normoxic conditions and stabilised by hypoxia.[52] Upon activation HIF-1 binds to the hypoxia responsive element (HRE), thereby promoting the transcription of numerous genes including VEGF and the genes encoding for the glucose transporters. Several cofactors are involved in the transcriptional regulation of the various target genes.

Under normoxic conditions two inhibitory pathways of HIF-1α are essential. Prolyl hydroxylases (PHD's) hydroxylate proline sites in the oxygen-dependent degradation domain (ODD) of the HIF-1α protein. This enables binding of the von Hippel Lindau protein (VHL), which leads to the proteasomal degradation of HIF-1α. Under hypoxic conditions hydroxylation does not occur, leading to accumulation of HIF-1. The second main inhibitory pathway of HIF-1α is factor inhibiting HIF-1 (FIH-1). FIH-1 hydroxylates the C-terminal transactivation domain (CAD) of HIF-1α, which inhibits binding of p300/CBP to the HIF-1 complex, which is a co-factor necessary for transcription. Besides the oxygen-dependent activation of HIF-1α, certain receptors of the tyrosine kinase family, like

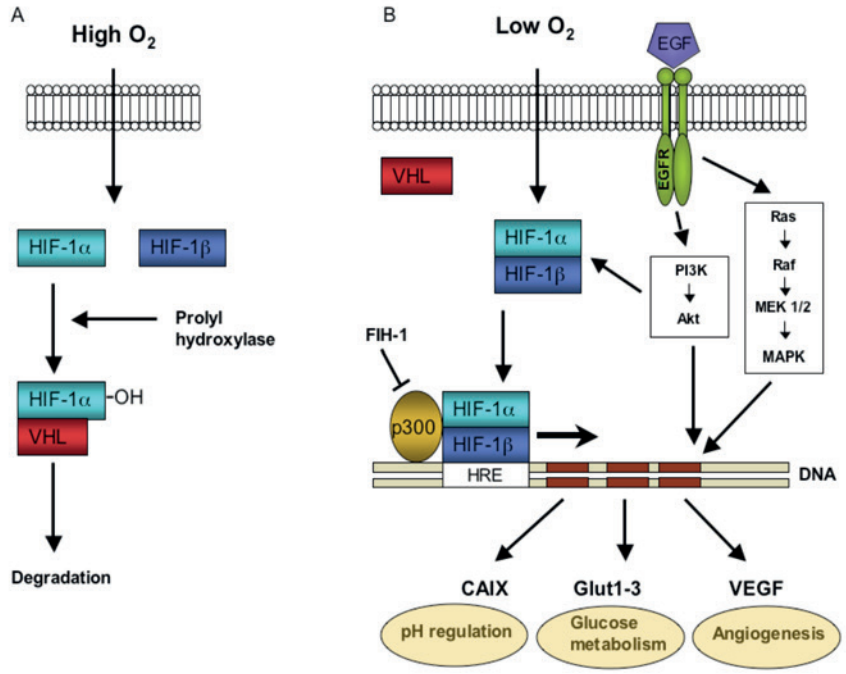

Fig. 4 Schematic representation of the HIF-1 pathway. Under normoxic conditions HIF-1α is hydroxylated and rapidly degraded (**A**). Under hypoxic conditions the HIF-1 complex is stabilised and initiates the transcription of its target genes. EGFR can activate HIF-1α in an oxygen-independent way (**B**).

insulin-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR) and HER2/neu, can activate HIF-1α in an oxygen-independent way. They regulate the transcriptional activity through the P13K/Akt/mTOR pathway.[53]

HIF-1α has gained interest as an endogenous hypoxia-related marker, after the discovery of its overexpression in a wide variety of malignant tumours.[54] Its significance as a prognostic factor for aggressive tumour behaviour has been proven in various types of cancer; clear cell carcinoma, ovarian carcinoma, gastric carcinoma, breast cancer, soft-tissue sarcoma, bladder cancer, head and neck cancer, rectal, lung cancer and cervical carcinoma[55], although some studies show an opposite effect.[56,57] However, the correlation between HIF-1α expression and oxygen-electrode or pimonidazole measurements is weak.[58] Therefore, the value of HIF-1α quantification as a hypoxia assay remains questionable.

Given the widespread overexpression of HIF-1 and its influence on multiple cellular functions, it is a promising therapeutic target, although the heterogeneity of the gene response also makes it a complex target.[59] Drugs aimed at the HIF-1 pathway can intervene

in multiple ways; decreasing HIF-1α mRNA or protein levels, inhibiting DNA binding of HIF-1 or decreasing HIF-1-mediated transactivation.[60] So far, clinical studies show limited success of these approaches.

Carbonic anhydrase IX

One of the downstream targets of HIF-1 is carbonic anhydrase 9 (CA9). The upregulation of CA9 under hypoxic conditions is controlled by the HIF-1 binding at the hypoxia responsive element (HRE) in its promoter region (Figure 4). This is supported by the fact that in VHL-defective renal cell carcinoma, in which the inhibition of HIF-1 is lost, extensive overexpression of CAIX can be found.[61] CAIX is one of the fourteen members of the CA-family, existing of cytosolic, membrane-associated, mitochondrial and secreted carbonic anhydrases.[62] CAIX (or MN, or G250) is a membrane-associated enzyme with a zinc-containing extra-cellular catalytic domain. This highly active domain catalyzes the reversible hydration of carbon dioxide to carbonic acid: H2O + CO2 ↔ H+ + HCO3-. It is involved in the respiratory gas exchange and acid-base balance, maintaining the intracellular pH and lowering the extracellular pH. CAIX can form a close interaction with certain ion transport systems, the so-called metabolons. The association with the bicarbonate transporter is recently established, affirming the role of CAIX in ion-transport and electrolyte-secretion.[63]

CAIX is only limitedly present in normal tissue; it is found in gastric mucosa, small intestine and muscle. The overexpression of CAIX is demonstrated in different types of cancer.[64] The CAIX-positive cells are mainly present in the perinecrotic areas of a tumour, in contrast to tumours with inactivated VHL, where a more general staining pattern is observed.[65] The expression shows overlap with the staining pattern of pimonidazole, but a strong correlation is not present.[45,66] There is no correlation between the amount of CAIX and direct oxygen measurement with the needle electrode in cervical carcinoma.[67] Notwithstanding, CAIX has proven to be a prognostic marker in various tumours, as high CAIX expression is associated with worse locoregional control and overall survival.[62] Especially the combination of CAIX and a proliferation marker such as IdU or Ki67, showed encouraging results. These markers identify cells that are proliferating under hypoxic conditions, perhaps the most crucial subpopulation of tumour cells.[26,68] In breast cancer, a quantifying assay of CAIX could identify patients who would respond least to adjuvant treatment, demonstrating its predictive value as well.[69]

Blocking the function of CAIX, resulting in an extracellular rise in pH, can be an interesting approach in cancer treatment. An acid tumour microenvironment can add to increased chemoresistance, decreasing the uptake and consequently the cytotoxicity of chemotherapeutic agents and enhances metastatic potential.[70] Specific inhibitors of CAIX have been developed, of which the sulphonamides are most promising.[71] Furthermore, a few monoclonal antibodies to block CAIX are under investigation in renal cell carcinoma,[72] a tumour type characterised by a high CAIX-overexpression.

Glucose transporters

Another group of genes that is upregulated in hypoxic conditions is that of genes encoding for the glucose transporters (GLUTs). These transmembrane glycoproteins are omnipresent in normal tissue, facilitating glucose transport across the cell membrane. Malignant tumours generally have a higher rate of metabolism, are more dependent on glycolysis as an energy source (Warburg effect) and therefore have a higher glucose need. Under hypoxic conditions the cell's demand for glucose increases as the anaerobic glycolysis becomes even more important. This also involves recruitment and overexpression of the glucose transporters in many malignant (hypoxic) tumours. The two glucose transporters most associated with invasive cancer are GLUT-1 and GLUT-3, being overexpressed in cervical carcinoma, head and neck cancer, colorectal and bladder cancer.[73] A third participant has been discovered more recently in breast and prostate cancer: GLUT-12.[74]

GLUT-1 as an endogenous hypoxia-related marker has been validated by Eppendorf histography, albeit with a weak correlation. Moreover, the comparison of immunohistochemical staining on biopsies of cervical carcinoma of GLUT-1 and pimonidazole showed similar staining patterns.[75] Several studies have assessed the applicability of the glucose transporters as prognostic markers. In cervical carcinoma high GLUT-1 expression has been associated with lower metastasis-free-survival[76], although in another study this correlation was not overtly present.[77] Additionally, in invasive bladder cancer, high GLUT-1 expression has been shown to be associated with poor survival.[78] GLUT-1 and GLUT-3 have been proposed as prognostic factors in head and neck cancer, but with equivocal conclusions.[79-81] Summarizing, GLUT expression has some prognostic potential in solid tumours, but it is not a very robust hypoxia-related marker.

Monocarboxylate transporters 1 and 4

Monocarboxylate transporters (MCT's) have been discovered to play an important role in tumour cell metabolism. Due to the high rate of glycolysis in malignant tumours, large amounts of lactic acid are produced. MCT's facilitate the uptake and excretion of monocarboxylates, like lactate and pyruvate, and act as monocarboxylate-proton symporters.[82] MCT4 is a low-affinity / high capacity lactate transporter, which is abundantly present in highly glycolytic muscle cells. It is one of the many target genes of hypoxia-inducible factor 1 (HIF-1), hence upregulation occurs under hypoxic conditions.[83] MCT1 is a high-affinity, low capacity monocarboxylate transporter, found in normal tissues like the intestinal epithelium (executing an important role in organic acid absorption), the blood brain barrier, red blood cells and skeletal muscle cells. Its expression seems to be regulated by multiple signaling pathways, microenvironmental parameters, changes in substrate concentration and pH.[82] They are suggested to play an important role in the symbiosis between lactate-producing and lactate consuming tumour cells.[33]

Lactate dehydrogenase

A last key protein related to the metabolism of tumor cells is lactate dehydrogenase-5 (LDH-5), responsible for the conversion of pyruvate into lactate. LDH-5 is one of the target enzymes of HIF-1 and has been described to have a strong association with HIF-1α expression in tumor tissue sections.[84,85]

Targeting hypoxia

Given the complexity of the hypoxic response, various strategies have been devised to target hypoxic cells. The most straightforward strategy is increasing the oxygen availability. Furthermore, some therapies have been developed using hypoxic cell specific toxins, others are aimed at decreasing their resistance to radiotherapy. Another field of research involves the development of drugs that target proteins involved in the hypoxic response or angiogenesis, but that is beyond the scope of this thesis.

ARCON (accelerated radiotherapy with carbogen and nicotinamide)

One of the first attempts to overcome hypoxia involved delivery of radiotherapy in hyperbaric oxygen chambers. In head and neck and cervical cancer patients treated with hyperbaric oxygen showed improved local tumour control and survival.[86] This treatment was eventually abandoned as hypoxia-modifying therapy due to the complex technique and poor patient compliance.

The next step was to combine radiotherapy with normobaric oxygen or carbogen (98% oxygen + 2% carbon dioxide) breathing. ARCON combines accelerated radiotherapy with carbogen breathing and nicotinamide, a vasoactive agent, counteracting both diffusion-limited and perfusion-limited hypoxia (Figure 5).[87] To limit clonogenic repopulation during therapy, the overall duration of radiotherapy is reduced by delivering multiple fractions per day.

In a phase II trial of head and neck cancer it demonstrated a substantial therapeutic effect in laryngeal carcinoma, notably in the more advanced tumour stages. The 3-year local control rate was 84% for T4 tumours, compared to around 50% in published series with radiotherapy alone.[88] This study demonstrated not only a prognostic value but also the predictive potential of the pimonidazole assay. An improvement in locoregional control with ARCON was observed only in patients with hypoxic tumours as assessed by pimonidazole staining.

In 2001 a phase III randomized trial was initiated in patients with advanced laryngeal cancer.

The first results demonstrate that ARCON results in a high level of compliance and a toxicity profile, comparable to what is encountered after accelerated radiotherapy alone.[89]

Bladder cancer is the second tumour site in which the combination of radiotherapy with carbogen breathing and nicotinamide (CON) has shown a beneficial effect. A phase

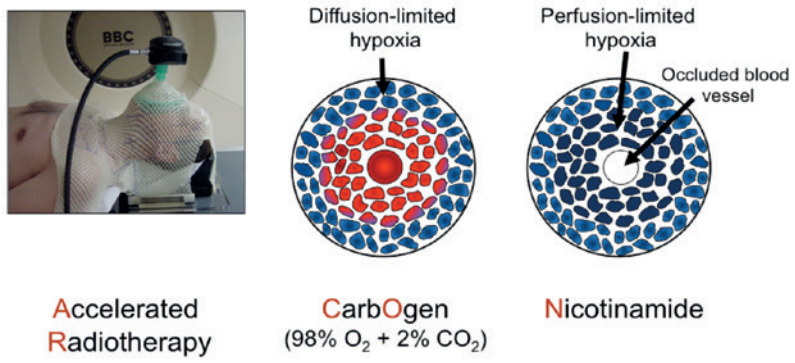

Fig. 5 The concept of ARCON therapy.

III trial with CON for bladder cancer shows an increase of the 3-yr local relapse-free survival from 43% to 54% and of the overall survival from 46% to 59%.[90] The late morbidity was similar in both treatment arms. The additional costs (carbogen, nicotinamide) of ARCON are very low, making it a cost-effective regimen.

Hypoxic cytotoxins

Bioreductive drugs, like tirapazamine (TPZ), exhibit a direct cytotoxic effect on hypoxic cells. In the absence of oxygen, reduction of TPZ takes place, forming a highly reactive radical, which induces DNA damage. TPZ has proven its therapeutic value in combination with cisplatin in a phase III randomized trial in stage IIIB and IV non-small-cell lung cancer, increasing the 1-year survival from 23% to 34%.(von-Pawel et al, 2000) Conversely, the combination of TPZ with cisplatin and radiotherapy in advanced head and neck cancer showed no improvement in failure-free survival and overall survival.[91] Unfortunately, no hypoxia-based selection of patients has been made in this trial, while a previous phase II study showed the effectiveness of this treatment regimen in patients with detectable hypoxia on a [^{18}F]-fluoromisonidazole positron emission tomography scan (FMISO-PET).[92]

Radiosensitisers

Nitroimidazoles are chemical compounds that mimic the radiosensitising effect of oxygen by inducing free-radical mediated double strand DNA breaks. The use of the first generation drug misonidazole, the most thoroughly documented of the nitroimidazoles, is obsolete in clinical practice as a radiosensitiser, due to its severe side effects. However, it still has the attention of some researchers as a PET-imaging compound at substantially lower doses. Of the other nitroimidazoles, nimorazole has proven its value as a hypoxic cell sensitising agent. In the Danish Head and Neck Cancer 5 study, in which 422 patients

with head and neck cancer were randomised, the locoregional control rate was significantly higher (49% versus 33%) when radiotherapy was combined with nimorazole compared to radiotherapy alone.[93] Nimorazole has not been adopted widespread as a standard treatment despite these promising results. The many negative studies with the older nitroimidazoles have contributed to this circumstance and a second randomized trial has never been performed.

To conclude, over the past years several hypoxia-based treatment strategies have been successfully applied in head and neck cancer patients. A meta-analysis shows improved locoregional control, disease-specific survival and overall survival with the addition of hypoxic modification to radiotherapy, emphasizing the importance of a hypoxia-modifying treatment regimen in head and neck cancer.[94]

Outline of the thesis

The aim of this thesis is to investigate the possibility to select patients with a laryngeal carcinoma for hypoxia-modifying treatment with ARCON based on immunohistochemical features. The relationship of the endogenous markers with hypoxia, as measured by pimonidazole, is investigated. Elucidating more about the response to hypoxia and establishing the value of the different markers may lead to the development of a predictive profile that enables the selection of patients for hypoxia-modifying treatments.

In **Chapter 2** the results of a large randomised trial in patients with laryngeal cancer comparing ARCON with accelerated radiotherapy alone are reported. The excellent local and regional control rates with ARCON in patients with a carcinoma of the larynx in a phase II study prompted this multicenter phase III study. A translational side study with the exogenous hypoxic marker pimonidazole adresses the issue of selecting patients based on the oxygenation status of the primary tumour.

As pimonidazole has the disadvantage of pre-biopsy administration, the quest for endogenous markers begins in **Chapter 3**, where a method is described to assess the colocalization of multiple markers in different subcellular compartments. Ki-67, a proliferation marker located in the nucleus, and CAIX, a membraneous hypoxia-related marker, are immunohistochemically stained to describe the parametric mapping method. The method is validated against manual scoring and results in multiple biopsies are compared.

Chapter 4 focuses on endogenous markers involved in cancer cell metabolism and their relation with hypoxia. Cancer cells often have a changed metabolism and depend more

often on aerobe glycolysis for their energy production than normal cells. Furthermore, under hypoxic circumstances cells will switch to anaerobe glycolysis. GLUT-1, LDH-5, MCT1 and MCT4 are involved in the glycolysis and CAIX in pH regulation. The master regulator of these proteins under hypoxia is HIF-1α. The correlation and colocalisation of these markers mutually and with pimonidazole is analyzed.

In **Chapter 5 and 6** the results obtained with the parametric mapping method are compared with the clinical data of the randomised trial to assess the prognostic and predictive value of these endogenous markers. Different patterns of CAIX expression are described in **Chapter 5** and the effect on prognosis and response to ARCON treatment is reported. **Chapter 6** concentrates on ARCON and proliferation. The prognostic value of Ki-67, an endogenous proliferation marker, is discussed and the benefit of hypoxic modification on tumors with a high and low proliferation rate.

A general discussion, based on the abovementioned chapters, is given in **Chapter 7** and **Chapters 8 and 9** provide an English and Dutch summary of the work.

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2

Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial

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Abstract

Purpose
To report the results from a randomized trial comparing Accelerated Radiotherapy (AR) with Accelerated Radiotherapy plus Carbogen and Nicotinamide (ARCON) in laryngeal cancer.

Methods
Patients with cT2-4 squamous cell laryngeal cancer were randomized to AR (68 Gy within 36-38 days) or ARCON (AR plus carbogen inhalation and nicotinamide).To limit the risk of laryngeal necrosis, ARCON patients received 64 Gy on the laryngeal cartilage. The primary endpoint was local control. Secondary endpoints were regional control, larynx preservation, toxicity, disease-free survival and overall survival. In a translational side study the hypoxia marker pimonidazole was used to assess the oxygenation status in tumor biopsies.

Results
From 04-2001 to 02-2008, 345 patients were accrued. After a median follow-up of 44 months, local tumor control rate at 5 years was 78% for AR versus 79% for ARCON (p=0.80) with corresponding larynx preservation rates of 84% and 87% (p=0.48). The 5-year regional control was significantly better with ARCON (93%) compared to AR (86%, p=0.04). The improvement in regional control was specifically observed in patients with hypoxic tumors and not in patients with well-oxygenated tumors (100% versus 55% respectively, p=0.01). AR and ARCON produced equal levels of toxicity.

Conclusions
Despite lack of benefit in local tumor control for advanced laryngeal cancers, a significant gain in regional control rate, with equal levels of toxicity, was observed in favor of ARCON. The poor regional control of patients with hypoxic tumors is specifically countered by ARCON treatment.

Introduction

Functional larynx preservation is a main goal when treating patients with loco-regional advanced laryngeal cancer.[1,2] The combination of radiotherapy with concurrent cisplatin demonstrated better larynx preservation and loco-regional control compared to conventional fractionated radiotherapy alone.[2] However, conventional fractionated radiotherapy is currently not considered the optimal strategy for head and neck cancer. Based on accomplishments of radiobiological research, new approaches in clinical radiotherapy have been developed to improve treatment outcome.

In head and neck cancer, tumor cell repopulation and tumor hypoxia are known factors determining radiation response. Accelerated Radiotherapy plus Carbogen and Nicotinamide (ARCON) is a strategy to counteract these resistance mechanisms.[3] To limit clonogenic repopulation during therapy, the overall duration of radiotherapy is reduced by delivering multiple fractions per day. This approach, referred to as accelerated fractionation (AR), has demonstrated superior loco-regional control rates in head and neck cancer.[4,5,6] ARCON combines accelerated radiotherapy with the inhalation of carbogen (98% O_2 + 2% CO_2) to decrease diffusion-limited hypoxia and the administration of nicotinamide, a vasoactive agent, to decrease perfusion-limited hypoxia.[7,8,9,10,11,12] Phase I and II trials have shown the feasibility and tolerability of ARCON for head and neck cancer and have produced promising results in terms of tumor control.[13,14,15] A favorable 5-year local control rate of 80% was obtained in a series of 79 T3 and T4 larynx carcinomas.[14]

This provided the basis for a multicenter trial randomizing patients with cT2-4 laryngeal cancer between AR and ARCON, of which the results are reported here. Data from this study on acute toxicity and compliance have been published recently.[16]

Additionally, in a translational side study the value of a tumor hypoxia assay to predict response to ARCON was assessed. For this purpose pimonidazole was used, a bioreductive chemical probe that forms protein adducts in viable hypoxic cells and can be visualized in tumor biopsies by immunofluorescence.[17]

Patients and methods

Study Design and Eligibility
This was an open-label, randomized phase III trial comparing AR with ARCON in laryngeal cancer. The trial was conducted under the auspices of the Dutch Head and Neck Cancer Group and the Dutch Cancer Society (KWF) in 7 centers in 2 countries (Table 1).

Eligibility was assessed by a multidisciplinary head and neck oncology team. Diagnostic workup consisted of full history and physical examination, blood cell count and blood biochemistry, laryngoscopy under general anesthesia with biopsy taking, CT- or MR-imaging of the larynx and neck, ultrasound-guided fine needle aspiration of

suspect lymph nodes and chest X-ray. All patients over the age of 18, WHO performance status ≤1 and squamous cell carcinoma of the larynx and the following clinical stage (TNM-classification, UICC 1997), were considered for this study: T2 glottic carcinoma with impaired cord mobility or subglottic extension, T2 supraglottic carcinoma with invasion of the mucosa of the base of tongue or vallecula or invasion of the medial wall of the piriform sinus, T3-4 glottic or supraglottic carcinoma and any N-stage but M0.

Exclusion criteria included prior or concurrent treatment for this tumor, severe stridor with impossibility for adequate debulking of airway, impaired renal and/or hepatic function (creatinine > upper normal limit, ASAT/ ALAT >1.5 times upper limit), use of nefrotoxic or anti-convulsant medication that could not be discontinued and a history of malignancy during the previous 5 years (with exception of basal cell carcinoma of the skin, carcinoma in situ of the cervix or superficial bladder cancer).

Approval for the study was obtained from the Radboud University Nijmegen Medical Centre research Ethics Committee with ratification from each centre before start. Written informed consent and completed quality of life questionnaire were obtained before randomization.

Sample Size

The target sample size was 344 patients (n= 172 per arm), determined to provide 80% power to detect a difference of 60 vs. 75% in local control rate at 2 years for AR vs. ARCON, respectively, allowing for dropouts and a significance level of 0.05 (two-sided log-rank test). Follow-up of 2 years after the last inclusion, was respected before data analysis.

Randomization and masking

Patients fulfilling enrollment criteria were centrally randomized by phone at the IKO (Integraal Kankercentrum Oost) trials office. Treatment arm assignments (AR vs. ARCON) were stratified for tumor site (glottic vs. supraglottic) and institution. A dynamic allocation method was used to avoid imbalance of treatment assignment within an institution. Randomization took place after all study investigations and no longer than 4 weeks prior to the anticipated start of treatment. Data were unmasked.

Treatment

A CT-scan in treatment position with immobilization device was used in all the patients. The initial radiation planning target volume encompassed the primary tumor and the lymph node levels II, III and IV in case of cN0. Level V and VI were included in case of cN1-3 and >1.0 cm subglottic extension, respectively.[18] The boost planning target volume encompassed the primary tumor volume and the macroscopic involved lymph nodes. A total dose of 44 Gy in 22 daily fractions of 2 Gy was prescribed followed by a boost dose of 24 Gy in twice daily fractions of 2 Gy with a minimum interval of 6 hours between the fractions. Dose specification and dose homogeneity requirements were according to

Table 1 Patient Demographics and Clinical Characteristics

Demographic or Clinical Characteristic	AR (n = 174)		ARCON (n = 171)	
	No. of Patients	%	No. of Patients	%
Age, years				
Median	60		61	
Range	38–88		41–84	
Sex				
Male	136	78	142	83
Female	38	22	29	17
Performance status				
0	140	80	137	81
1	34	20	34	19
Site of the primary tumor				
Supraglottic	100	57	97	56
Glottic	74	43	74	44
T stage				
T2	67	38	55	32
T3	80	46	95	56
T4	27	16	21	12
N stage				
N0	117	67	116	68
N1	20	12	23	13
N2a	4	2	7	4
N2b	10	6	5	3
N2c	23	13	20	12
N3	0	0	0	0
Participating institutions*				
Radboud University Nijmegen Medical Centre, Nijmegen	75	43	76	44
University Medical Center Utrecht, Utrecht	38	22	39	23
VU University Medical Center, Amsterdam	21	12	20	12
University Medical Center Groningen, Groningen	15	9	13	8
Maastricht University Medical Centre, Maastricht	15	9	13	8
Leiden University Medical Center, Leiden	8	4	9	5
Mount Vernon Hospital, Northwood, United Kingdom	2	1	1	0

Abbreviations: AR, accelerated radiotherapy; ARCON, accelerated radiotherapy plus carbogen and nicotinamide; VU, Vrije Universiteit.

* All institutions, except Mount Vernon Hospital, are in the Netherlands.

Report 50 of the ICRU.[19] The overall treatment time was 36-38 days. Because a decrease in radiation tolerance was observed for cartilage and spinal cord in earlier studies with hypoxic sensitization, the total dose to the arytenoid cartilage and the spinal cord in the ARCON arm was limited to 64 and 40 Gy, respectively.[20,21]

Patients allocated to the ARCON arm received carbogen (98% O_2 + 2% CO_2, 4 minutes before and during daily fractions) and oral nicotinamide (60 mg/kg, 1-1.5h before each fraction) concurrently with radiotherapy.[22,23] During the boost nicotinamide was given only before the first fraction of the day. To prevent nausea, domperidone (10 mg, thrice-daily dosage) was given.

Hypoxia marker side study

After informed consent, patients received pimonidazole (Hypoxyprobe-1; Natural Pharmacia International, Belmont, MA) intravenously (500 mg/m^2) two hours before biopsy taking. Biopsies were snap frozen in liquid nitrogen, immunohistochemically stained and semi-automatically analyzed as described earlier.[24] Of each biopsy, one complete section was analyzed to define the hypoxic fraction, i.e. the area positive for pimonidazole relative to the total tumor area. Based on previous pimonidazole marker studies, a cut-off value of 2.6% was used to dichotomize between well-oxygenized and hypoxic tumors.

Monitoring During Treatment and Follow-up Evaluations

Acute radiation toxicities were graded according to an earlier validated list of toxicity criteria (online only table 1).[13] Any other side effects felt to be related to carbogen or nicotinamide and the reason for interruption or discontinuation were recorded as well. After resolution of the acute radiation toxicities, follow-up visits took place every 2, 3, 4 months during the first, second and third year respectively, then every 6 months for another 2 years. The larynx was assessed by fiberoptic or indirect laryngoscopy. Regional control was assessed by palpation of the neck. On suspicion of nodal recurrence an ultrasound with fine needle aspiration cytology was performed. Recurrences were cytologically or pathologically confirmed and documented by CT-scan or MRI. Eligibility for salvage neck dissection was assessed by a multidisciplinary head and neck oncology team. Severe adverse events were defined as either events that are fatal, life-threatening or resulting in permanent disability or any late toxicity like deep mucosal necrosis, cartilage necrosis or osteoradionecrosis requiring surgery.

Endpoints and Statistics

The primary endpoint of the study was local tumor control. Local control was taken as freedom of first recurrence at the primary tumor site. Secondary endpoints were regional control, larynx preservation, toxicity, quality of life, disease-free survival (DFS) and overall survival (OS). Regional control was defined as freedom of first regional recurrence. Disease-free survival was defined as the time to a local or regional recurrence, distant metastasis, or death from any cause. Overall survival was defined as time to death. For larynx preservation, treatment was considered to have failed on the date laryngectomy was performed.

Statistical analyses were performed using SPSS 16.0.1. Survival estimates were obtained using the Kaplan-Meier method and all analyses were based on intent-to-treat policy. All intervals were calculated from the date of randomization and censored after 60 months or at last follow-up. Differences were compared using the log-rank test and hazard ratios (HR) and their 95% confidence interval (CI) were obtained using the Cox proportional hazards model. Differences in prevalence of worst grade acute and late toxicities between both treatment arms were compared using the Chi-square test.

Results

Patient Characteristics and Protocol Compliance

Between April 2001 and February 2008, 345 patients were randomized to either AR or ARCON (Figure 1). The median follow-up time was 44 (range 18-103), 55 and 60 months for the whole group and for patients still alive receiving AR and ARCON, respectively. Patient demographics and clinical tumor characteristics were well balanced without significant differences between the groups (Table 1). Compliance to radiotherapy, carbogen breathing and nicotinamide intake was high.[16] Radiotherapy was delivered as planned to 173 (99%) AR and 169 (99%) ARCON patients and was completed within the specified time of 38 days for 168 (97%) AR and 163 (96%) ARCON patients. Full compliance to carbogen breathing, nicotinamide intake and the combined treatment (ARCON) was 86%, 80% and 76%, respectively.

Tumor Control and Survival

Five-year larynx preservation rates were 84% and 87% for AR and for ARCON (p = 0.48). There was no significant difference in local control rate: 80% and 78% for AR versus 83% and 79% for ARCON at 2 and 5 years, respectively (HR 0.94; CI 0.58–1.52; p=0.80; Figure 2A). The 2- and 5-year regional control rates were significantly better with ARCON (88% and 86% for AR versus 95% and 93% for ARCON, respectively (HR 0.46; CI 0.22-0.97; p=0.04; Figure 2B). No significant differences were found for DFS (HR 0.75, CI 0.50–1.13, p=0.16; Figure 2C) and OS (HR 1.03; CI 0.73–1.46; p=0.86; Figure 2D). In exploratory subgroup analysis we did not find significant differences in 5-year local control rate between treatment arms when stratified for T-stage or laryngeal subsite.

Salvage laryngectomy was attempted in 29 of 35 AR and 21 of 28 ARCON recurrences. Salvage neck dissection was attempted in 12 of 21 and 8 of 12 patients treated by AR and ARCON, respectively. The ultimate 5-year local and regional control rates, including salvage therapy were 94% vs. 92% (p=0.60) and 92% vs. 98% (p=0.21) for AR and ARCON, respectively.

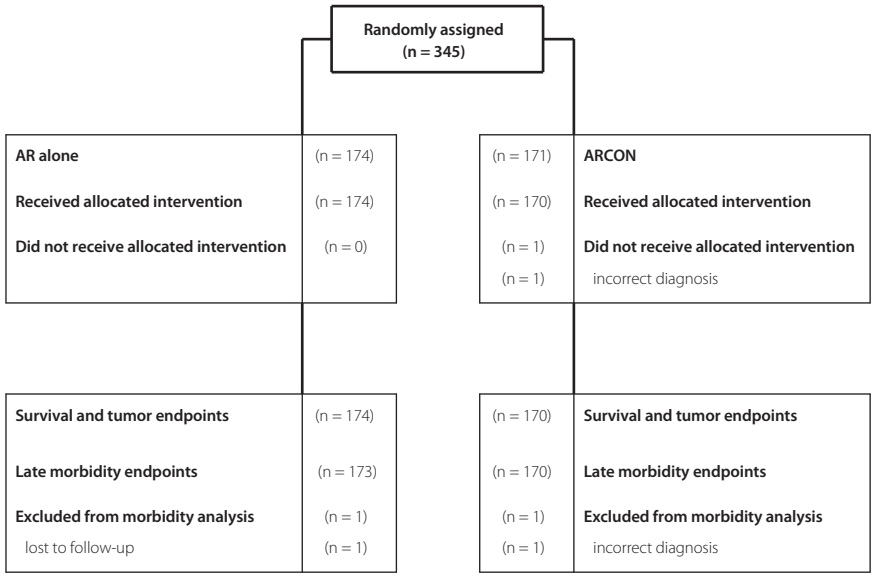

Fig. 1 CONSORT flowchart. AR, Accelerated Radiotherapy; ARCON, Accelerated Radiotherapy plus Carbogen and Nicotinamide.

Translational side study

Two centers participated in the hypoxia marker study and included 79 patients. The hypoxic fraction, as defined by pimonidazole staining, varied from 0-19.4% with a median value of 1.5%. There was no ascertainable benefit from ARCON with regard to local control, neither in the well-oxgenated tumors, nor in the hypoxic tumors (Figure 3A,B). However, regional control in the group with a high hypoxic fraction was significantly improved with ARCON compared to AR (100% vs. 55%, p=0.01), while no difference between the treatment arms was observed in the group with a low hypoxic fraction (96% for ARCON vs. 92% for AR, p=0.70) (Figure 3C,D). Patients with hypoxic tumors had a substantially higher five-year DFS in the ARCON arm (86%) compared to AR (40%), although the difference did not reach the significance level (p=0.08) (Figure 3E,F). No DFS benefit from ARCON was observed in patients with well oxygenated tumors (80% for ARCON vs. 77% for AR, p=0.80).

Toxicity

Toxicity data have been described extensively in a recent publication.[16] Between both treatment arms (AR vs. ARCON) no statistically significant difference was observed for incidence of acute skin reactions (moist desquamation: 56% vs. 58%, p=0.80), acute mucosal reactions (confluent mucositis: 79% vs. 85%, p=0.14) and symptoms related to

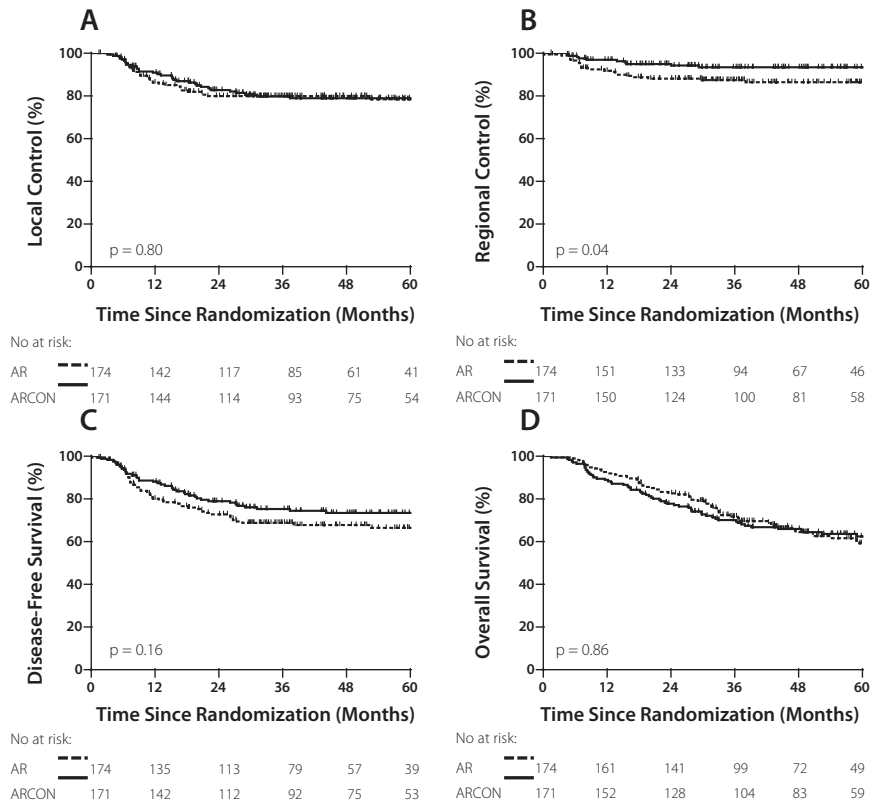

Fig. 2 Kaplan-Meier curves for local control (**A**), regional control (**B**), disease-free survival (**C**) and overall survival (**D**) comparing accelerated radiotherapy only (AR) or accelerated radiotherapy plus carbogen and nicotinamide (ARCON). Log-rank P values are shown and number of patients at risk against yearly intervals.

acute mucositis (severe pain on swallowing: 53% vs. 58%, p=0.37; nasogastric tube feeding: 28% vs. 28%, p=0.98, narcotic medicines required: 58% vs. 58%, p=0.97). There was a small but statistically significant difference in median duration of confluent mucositis in favor of AR (2.0 vs. 3.0 weeks, p=0.01).

Analysis of late radiation morbidity did not reveal significant differences between AR and ARCON for skin and subcutaneous tissues (severe teleangiectasia: 8% vs. 8%, p=0.56; severe subcutaneous fibrosis: 7% vs. 9%, p=0.26; severe subcutaneous edema, 8% vs. 4%, p=0.09) and mucous membranes (mucosal ulceration, 8% vs. 6%, p=0.36; nasogastric tube feeding, 6% vs. 6%, p=0.61), respectively. Twelve patients in the AR group and 6 patients in the ARCON group received a tracheostomy for severe edema with dyspnea

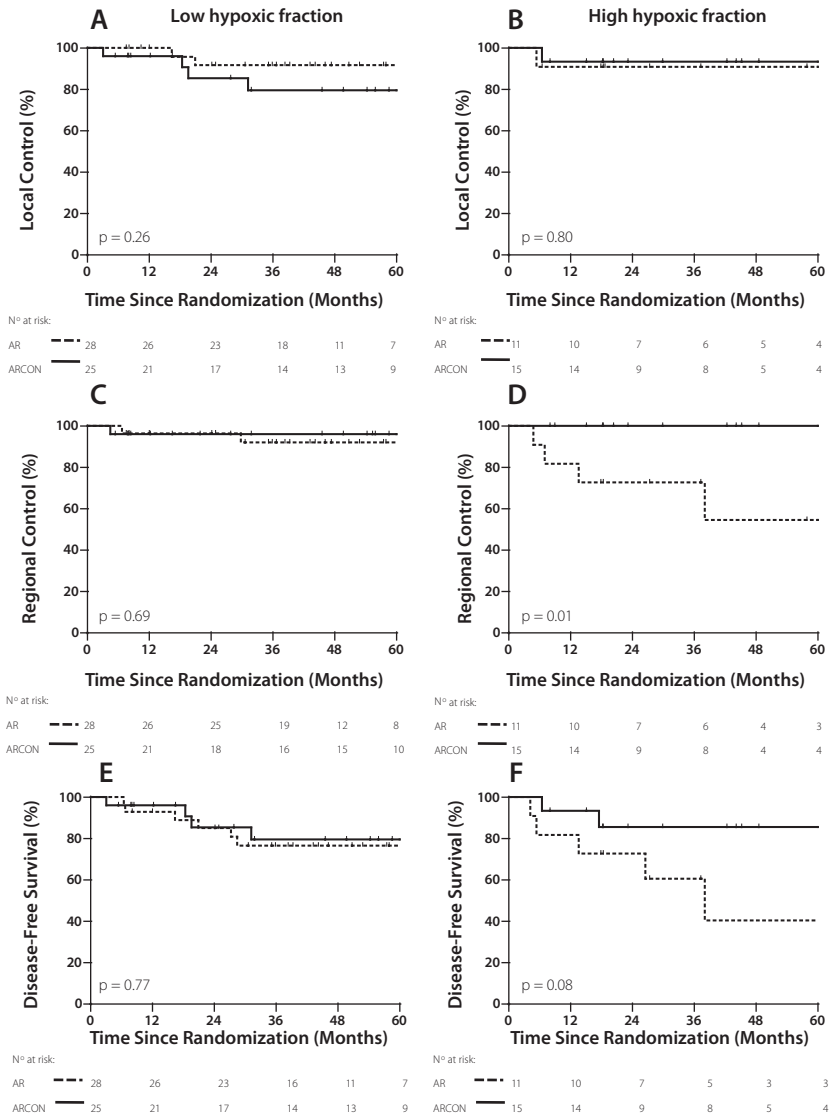

Fig. 3 Local control (**A**,**B**), regional control (**C**,**D**) and disease-free survival (**E**,**F**) by oxygenation status of the primary tumor and treatment arm. Log-rank P values are shown and number of patients at risk against yearly intervals.

and/or stridor. All patients who developed a cartilage necrosis could be managed conservatively. Four of them did receive a tracheostomy (n=1 for AR, n=3 for ARCON) and there was no need for laryngectomy. One year after diagnosis 9% of the patients with the larynx in situ assigned to AR and 7% of patients in the ARCON group could swallow only soft foods or liquids and 3% of patients in both groups needed nasogastric tube feeding (p=0.88).

Severe adverse events

One patient (AR) died from a cardiac arrest during treatment. The cause of death was unrelated to treatment. Another patient (ARCON) died one day after the end of treatment due to a gastric bleeding. He was using diclofenac for abdominal pain. Causality with nicotinamide was considered uncertain for this patient. Upper airway obstruction during radiotherapy, requiring tracheostomy, occurred in 4 patients treated by AR and in 1 patient with ARCON. Furthermore, in the ARCON arm one patient developed a renal insufficiency and sepsis and one patient was admitted to the hospital with severe bleeding from the tumor.

Discussion

The addition of carbogen and nicotinamide to a schedule of accelerated radiotherapy produces a significant gain in regional control relative to accelerated radiotherapy alone but no benefit in local control in patients with T2-T4 laryngeal cancer. This improvement in regional control can be entirely attributed to the patients with hypoxic tumors as assessed by pimonidazole staining.

Although there was no improvement in local control with ARCON, the result in the experimental arm was consistent with the 80% local control rate at 5 years obtained in the preceding phase II ARCON trial.[14] Unexpectedly, the local control rate in the control arm was higher than the 63-76% and 43-50% local control rates observed for T3 and T4 laryngeal cancers treated by hyperfractionated and accelerated regimens reported at the time of onset of this study.[25,26] A possible explanation might be stage migration due to better diagnostic imaging, especially when comparing the results of a prospective study with retrospective reports of 15 years ago. In the current study no significant differences between T-stages were observed. With conventional fractionation schedules local control rates are in the order of 67-80%, 30-77% and 26-52% for T2, T3 and T4 tumors, respectively, indicating that patients with more advanced local disease profit most from accelerated fractionation.[27,28,29,30,31]

Because the purpose of this study was to arrive at improved tumor control with no increase of late laryngeal toxicity, a dose reduction to 64 Gy for the larynx was prescribed for patients in the ARCON arm. This dose reduction was based on a decrease in radiation

tolerance in the order of 10% observed for cartilage when radiotherapy was given in hyperbaric oxygen.[20] In the current study a similar effect was expected from normobaric carbogen with nicotinamide. This 4 Gy absolute dose difference on the larynx is a possible explanation for the lack of additional benefit in local tumor control with ARCON as compared to AR alone. Based on clinical data, dose-response curves have been constructed for head and neck carcinomas.[32] From these, dose-response gradients (gamma value) have been derived as a measure of the steepness of the curve. Typical gamma values for larynx carcinoma range from 1.5-2.5. This means that for each percent increment in dose, the probability of controlling the tumor will increase by approximately 2 percentage points. Given the same local control but with 4 Gy less dose, an enhancement of tumor control probability of 10-15% can be derived when carbogen and nicotinamide are combined with radiotherapy. Thus, although a dose difference of 4 Gy seems modest, the corresponding difference in cure rate is significant.

In contrast to the primary tumor, the dose delivered to the lymph node metastases in the current study was similar (68 Gy) in both arms. The addition of carbogen and nicotinamide did lead to a significant improvement of 8% in regional control rate. This finding is consistent with the results of a recent systematic review and meta-analysis of hypoxia modification in head and neck cancer in which an absolute risk reduction of 8% for loco-regional control was observed.[33]

The high rate of local and regional control rates in both treatment arms of the current trial and the potential to perform successful salvage surgery in case of recurrence explains the lack of benefit in overall survival, similar to what is observed after conventional radiotherapy with concurrent cisplatinum in the RTOG 91-11 study.[2]

In the current study, long-term swallowing function was similar for AR and ARCON. One year after treatment, swallowing was limited to soft foods or liquid in 9% and 7% of patients respectively. This is lower than the 23% reported in RTOG 91-11.[2] However, direct comparison is difficult because of potential bias, e.g. RTOG 91-11 included more supraglottic tumors. Furthermore, unlike the current study, planned neck dissection was performed in case of cN2a or cN2b disease. Neck dissection after chemoradiotherapy has been demonstrated to increase the risk of long-term dysphagia and laryngeal dysfunction.[34]

Recently published data from this study demonstrate that ARCON results in a high level of compliance and a toxicity profile, comparable to what is encountered after accelerated radiotherapy alone.[16,35] The 8% improvement in regional control and excellent larynx preservation rate support the evidence that ARCON can achieve a therapeutic gain. Probably, head and neck tumors at other subsites, where the laryngeal cartilage is not the dose-limiting organ, e.g. oropharynx, will profit more from ARCON, especially those with advanced nodal stage. The recently published results from a phase III trial with carbogen and nicotinamide for bladder cancer confirm the value of this hypoxia-modifying approach.[36] Radiotherapy with carbogen and nicotinamide produced a significant

improvement in local relapse rate, bladder conservation rate and overall survival. The additional costs (carbogen, nicotinamide) of ARCON are very low, making it a cost-effective regimen.

The present study demonstrates the importance of a proper patient selection that is based on the mode of action of the treatment under investigation, as only patients with hypoxic tumors did profit from ARCON therapy and no gain was seen in patients with well-oxygenated tumors. This finding strongly supports the notion that assessment of the tumor oxygenation status provides a powerful selection tool for hypoxia-modifying treatment on an individual patient basis. The inclusion of unselected patient populations may be an important reason for the modest improvements generally reported by studies employing hypoxic modification.[33,37] Further translational research linked to this study is currently investigating the predictive value of a hypoxia-associated gene signature.

Conclusion

The use of ARCON in stage II-IV laryngeal cancer produced a significant gain in regional control rate compared to AR, with similar acute and late toxicity. There was no difference in local control between the treatment arms. Translational research employing a hypoxia marker assay demonstrates that proper patient selection based on tumor biology is the key to the success of this approach.

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3

Parametric mapping of immunohistochemically stained tissue sections; a method to quantify the colocalization of tumor markers

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Abstract

Purpose

Automated analysis of immunohistochemically stained tissue sections is of great importance in cancer research to detect tumor-specific prognostic markers and make therapy decisions. Here, an automated quantitative analysis is presented to assess the colocalization of CAIX, a membrane-bound hypoxic marker and Ki-67, a nuclear proliferation marker.

Methods

Tissue sections of 104 biopsies from 89 patients were stained for CAIX and Ki-67 with diaminobenzidine and haematoxylin counterstain. Image scans of whole tumor sections were recorded and image maps were created with parametric mapping to quantify the markers and assess the colocalization.

Results

The fraction of CAIX showed a range of 0 – 93%. The interobserver correlation and the correlation between manual scores and automated analysis were both very strong ($r_s = 0.96$, $p < 0.0001$, and $r_s = 0.97$, $p < 0.0001$). The labelling index of Ki-67 exhibited a range of 0 –42% with less strong interobserver and manual to automated analysis correlations ($r_s = 0.90$, $p < 0.0001$, and $r_s = 0.71$, $p < 0.0008$). The relative tumor area positive for both markers varied from 0 – 76%.

Conclusion

Parametric mapping of immunohistochemically stained tumor sections is a reliable method to quantitatively analyze membrane-bound proteins and assess the colocalization of various tumor markers in different subcellular compartments.

Introduction

In cancer research immunohistochemistry is an important technique to characterize protein expression in tumors, while preserving tissue morphology. By clarifying aberrant overexpression of proteins, prognostic factors can be discovered and highly specific therapies can be developed. A well-known example of such a tumor-specific marker is Her-2/neu in breast cancer.[1]

Immunohistochemistry is based on an antibody-antigen interaction, combined with various detection techniques.[2] A frequently used method is based on a peroxidase-catalyzed reaction. The enzyme is conjugated to the primary (direct method) or secondary (indirect method) antibody and by adding diaminobenzidene (DAB) the labelled antibody is visualized by brown staining. The staining can be accentuated by counterstaining with haematoxylin, which stains the background tissue blue.

The interpretation of immunostains has made a rapid development from manual counting to fully automated techniques for image capture and analysis.[3,4,5] Although visual evaluation cannot be replaced entirely by these methods, automated image analysis has some major advantages. In clinical practice for instance, it increases throughput and reproducibility.[6] Furthermore, in recent research even localization of protein expression at the subcellular level was achieved[7], illustrating the additional possibilities of automated analysis over manual counting. Finally, manual interpretation is subject to high interobserver variability and is semi-quantitative at best[5], whereas in a research setting quantitative information on a continuous scale can be of great importance.

Several systems for analysis have been described, varying in software, threshold selection, colour format and algorithms.[8] The reports on quantitative automated image analysis focus on various parameters, like vascular density[9], nuclear staining or intensity of staining[4,10]. Automatic quantification of the fraction of a membrane-bound protein poses some difficulties, although automated filling operations are applied to facilitate this analysis[11,12]. Intensity-based quantification of a membrane-bound protein has been described using a membrane isolation algorithm.[13]

The automated evaluation of multiple markers is a challenging issue. Most of the automated analysis methods that are available concentrate on one marker at a time. Whereas, especially in tumor cells, the co-localization of different markers can provide valuable information. The exact co-localization on a cellular level is difficult to assess when the markers have a different intracellular location, e.g. nuclear and membranous. Here, the absence of overlap on pixel level renders a binary image map comparison[14] unsuitable. Parametric mapping can solve this problem by creating an analysis grid defining square regions of multiple pixels. With this technique the whole image is subdivided in small squares. The information of all immunopositive objects or pixels within a square can be translated into numerical data, e.g. cell number, mean staining intensity, number of positively stained pixels etc. Different parameters can be combined in one value, like

presence or absence of colocalization, and can be assessed for the whole tissue section. Thus, colocalization can be determined on a near-cellular level instead of a pixel level. This technique has been described previously in a different field of research for the assessment of the immunopositive cell density in the rat brain.[15] An advantage of this method is the retainment of intensity values, reflecting the concentration of the stain in the tissue, which would be lost when performing binary analysis.

In this study we applied the parametric mapping method to examine two important features of malignant tumors: proliferation and hypoxia. Proliferation is an important prognostic factor in many types of cancer. Highly proliferating tumors are associated with more aggressive biological behaviour and a worse prognosis.[16,17] Hypoxia is another adverse prognostic factor, making tumor cells more resistant to therapy as well.[18] A combination of these two features could identify a subpopulation of tumor cells that is highly relevant for treatment responsiveness.[11] Several hypoxia-related markers are available to measure the amount of hypoxia in tumors.[19,20] For the research presented here, carbonic anhydrase IX (CAIX) was used, a membrane-bound endogenous hypoxia-related marker to assess the feasibility of this method. In future research other biologically connected markers can be investigated, for example epidermal growth factor receptor (EGFR) and Ki-67.

In this study a quantitative computer-assisted analysis of immunohistochemically stained sections using parametric mapping is described and evaluated for CAIX, the membrane-bound protein, and for the colocalization of CAIX with Ki-67, a nuclear proliferation marker, to identify the proliferating hypoxic subpopulation of tumor cells. The effect of varying the resolution of the analysis will be presented and the method will be correlated to manual scoring. Finally, multiple biopsies from the same tumor will be scored to get an indication of the intratumor variability.

Materials and Methods

Samples

Biopsy material obtained for routine purposes of 103 patients diagnosed between 2001 and 2008 with advanced laryngeal carcinomas was retrieved from the pathology department of the Radboud University Nijmegen Medical Centre in the Netherlands. From some patients multiple biopsies of the same tumor were available. The biopsies were obtained for diagnostic purposes and had been fixed in formaldehyde and paraffin-embedded. The samples were cut in sections of 5µm. One section was stained for haematoxylin and eosin and two consecutive sections for Ki-67 and CAIX, both with a haematoxylin counterstain.

Immunohistochemistry

Sections were deparaffinised in Histosafe (clearing agent, Adamas, the Netherlands), rehydrated through a graded ethanol series and boiled for 30 minutes in antigen retrieval solution. After cooling for 25 minutes and rinsing in PBS, endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Then, sections were incubated with 5% normal donkey serum in PAD (primary antibody diluent, Abcam, UK) to block aspecific binding.

Sections were incubated at 4 °C overnight with mouse-anti-CAIX (E. Oosterwijk, department of Urology, Radboud University Nijmegen Medical Centre) diluted 1:25 in PAD or rabbit-anti-human Ki-67 (Abcam, UK) diluted 1:50 in PAD. Subsequently, the biotinylated secondary antibodies were applied; biotin-labelled-F(ab')$_2$-donkey-anti-mouse IgG (Jackson Immuno Research) for CAIX and biotin-labelled-F(ab')$_2$-donkey-anti-rabbit IgG (Jackson Immuno Research) for Ki-67, followed by ABC-reagent (Vector Elite kit, Vector Laboratories) for 30 minutes. Peroxidase activity was detected with diaminobenzidine (DAB). Finally, sections were counterstained with haematoxylin for 30 seconds and mounted with KP mounting medium (Klinipath, Duiven, the Netherlands). For negative controls, PAD was added without the primary antibody.

Image processing

A digital image processing system for fluorescence microscopy[21] was adapted to scan immunohistochemically stained sections using bright field microscopy. This system consisted of a monochrome CCD camera (Retiga SRV, 1392 x 1040 pixels) and a RGB filter (Slider Module; QImaging, Burnaby, BC, Canada) attached to a motorized bright field microscope (DM6000 Leica, Wetzlar, Germany). Whole tumor sections were scanned with a 10 x objective at 100x magnification using a Macintosh computer running IPLab for Macintosh (Scanalytics Inc., Fairfax, VA, USA), which controlled this motorized system and generated 24-bit colour composite images (pixel size 1,8 µm). For every scan session a separate background image was recorded from an individual microscopic field in one focus plane and used to construct composite background images corresponding in size with the tumor section scans.

To extract and separate the individual colours from the DAB (brown) and haematoxylin (blue) signals, the RGB linear unmixing module in the TRI2-software (P.R. Barber, R. Locke, R. Edens, S. Ameer-Beg, B. Vojnovic and J. Gilbey; Randall Division and Gray Institute) was applied using the "Absorption" mode with a nonnegative least squares algorithm[22]. This resulted in grayscale images in which the pixel values represent the concentration of a marker (Figure 1, second panel, pseudo-coloured).

The colours to which the images were unmixed were obtained from previously saved reference files. In these files colour information was stored from the blue (haematoxylin) and brown (Ki67, CAIX) signals and was selected in images that were acquired from single-stained control sections. During linear unmixing, the RGB colour image was corrected for the microscope illumination by using the 'background' image. For further processing of the grayscale images, cut off values for the DAB and haematoxylin signals

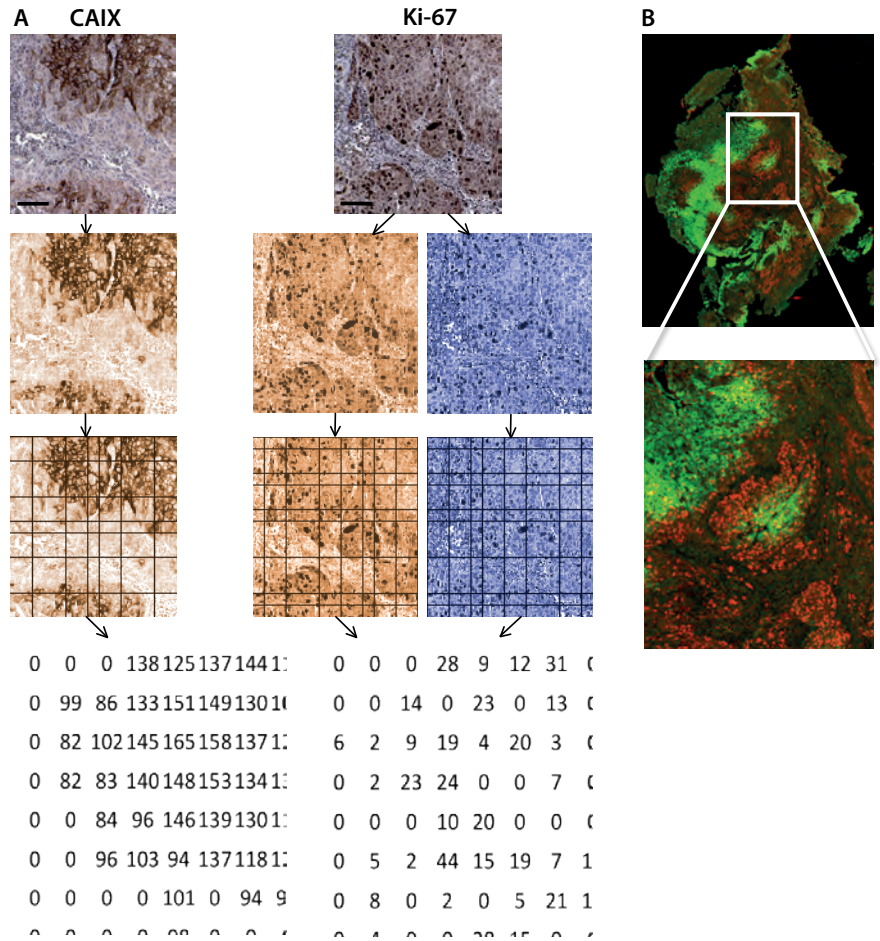

Fig. 1 (**A**) Overview of the process of image preparation and analysis. Example of an original DAB and hematoxylin stained tumor section for Ki-67 and CAIX (top) with the resulting pseudo-colored grayscale images after linear unmixing (second row). The third and fourth row show the conversion of immunopositive objects into numerical data by parametric mapping using square compartments of 20 x 20 pixels. Note the intensity values in the resulting CAIX image map and the labeling indexes in the Ki-67 image map. (**B**) A pseudo-colored image was constructed by merging the Ki-67 (red) and CAIX (green) images to check the match with a magnification of the area indicated by the white square. Scalebars: 100 mm.

were selected visually above the background staining for Ki-67. For CAIX, one threshold could be set for each staining series, based on the mean background of each section within the series. The cut off value was defined as the mean background + two times the standard variation. To be able to quantitatively compare the overlap of the Ki-67 signal and the CAIX stained areas in the consecutive images, CAIX images were rotated and shifted to fit as close as possible the Ki-67 images using the interactive "register" function in IPLab. A pseudo-coloured image composed of the corresponding grayscale images was used to check the match (Figure 1B). Large deviations (> 2 cells disparity) were corrected by refitting. In case of a fragmented or damaged tissue section, it was required to divide the image in two parts and match and analyse these parts separately.

Haematoxylin/eosin stained sections were used as a guide to manually delineate the tumor area on the Ki-67 image scan in IPLab, excluding normal tissue, necrotic areas and artefacts, creating a mask for image analysis.

Parametric mapping

As the markers investigated in this study are localized in different subcellular compartments, i.e. nucleus and membrane, a newly developed parametric mapping technique was applied to reduce the spatial information in the images. Hereto, all grayscale images were subdivided in small squares of 20 x 20 pixels, corresponding to a size of 36 µm x 36 µm (Figure 1A, third row). This size was chosen based on cell size and small deviations in the fitting of consecutive tissue sections. From the Ki-67 image and the corresponding haematoxylin image the labelling index of Ki-67 was calculated for every square, defined as DAB-positive area divided by the total haematoxylin-stained nuclear area (values between 0 and 100). This result was set as a new value in the corresponding square of a new image, creating a so-called parameter image map (Figure 1A, fourth row). In the corresponding CAIX image, the average intensity of the CAIX staining was measured for each square (values between 0 and 255) and set as a new value in the corresponding square of a second parameter image map. In all cases, only the pixels with a value above the preset cut off value were included. Objects (contiguous groups of pixels above the cut off value) smaller than 6 pixels were considered non-specific staining or cutting artefacts and were excluded from the analysis. Using these parameter image maps, the CAIX positive area and the Ki-67 labelling index for the whole section were calculated. The CAIX positive area (or CAIX fraction) was defined as the percentage of squares in the parameter image map with values higher than zero. For Ki-67, the labelling index of the whole section was calculated by averaging the values of all squares in the parameter image map.

The relationship between proliferation and CA-IX expression was analysed by determining the overlap for each biopsy. By combining the CAIX image map and the Ki-67 image map, every square has a value for CAIX and one for Ki-67. Overlap for the whole tissue section was defined by the percentage of squares having a value > 0 for both parameters, leaving the intensity values out of consideration in the first calculations.

To further evaluate this colocalization and explore the possibilities of this method, four classes were defined. Class 1 includes squares with CAIX intensity < 100 and Ki-67 labelling index < 25, representing colocalization of weak staining patterns for both markers. Class 4 represents strong colocalization with square values of CAIX ≥ 100 and Ki-67 labelling index ≥ 25. Class 2 (CAIX < 100, Ki-67 ≥ 25) and class 3 (CAIX ≥ 100, Ki-67 < 25) include the squares with one parameter high and one low.

To evaluate the effect of the size of the squares (or grid size) on the results, the analysis was repeated with a square size of 10 x 10 pixels (approximately one cell) and 40 x 40 pixels (approximately 16 cells) in a subgroup of 18 tumors.

Manual scoring

To compare manual assessment of both markers with quantitative digital analysis, two investigators (S.R. and W.P.) scored the area positive for CAIX on the microscope in a subgroup of 17 tumors, blinded for the result of the automated analysis. The whole tumor section was scored semiquantitatively per field of view at 100x magnification, the mean of all fields of view representing the final score . The labelling index of Ki-67 was scored in three fields of view in representative parts of the tumor section with a 400x magnification by counting the positive nuclei and the total number of nuclei in that field. Only dark brown stained nuclei were considered Ki-67 positive.

To validate the colocalization, the percentage of "true" colocalization and the percentage of mismatch were determined within the squares (Figure 5c). In 3 sections 200 squares were scored for CAIX-positivity, Ki-67-positivity and the presence of true colocalization.

Statistics

Statistical analyses were performed on a Macintosh computer using Prism 4.0 (Hearne Scientific software, Dublin, Ireland) software package. To assess the correlation between manual and computer-assisted scores and the inter-observer variation the Spearman correlation coefficient was calculated and Bland-Altman analyses were performed. Linear regression analysis was done to correlate the results of the different grid sizes with the manual score. P-values < 0.05 were considered significant.

Results

Biopsy material was obtained from 103 patients and from 15 of these patients two or more biopsies were available. All sections were evaluated on their suitability for analysis. Fourteen biopsies were excluded, because of the absence of invasive carcinoma or the poor quality of the sample. In total, 104 biopsies of 89 tumors were available for analysis. The tumor area of all sections ranged from 0.1 mm^2 to 26.4 mm^2 with a median value of 3.7 mm^2.

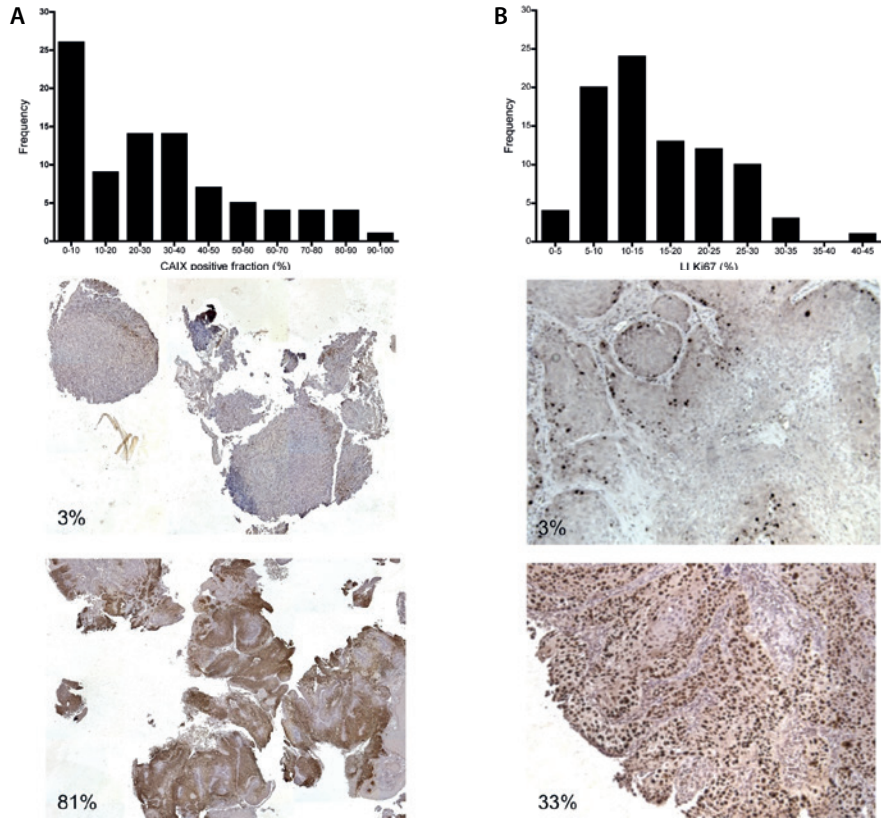

Fig. 2 Results of the automated single marker analysis for CAIX and Ki-67. Frequency distribution of the relative area positive for CAIX showed a range of 0 – 93% with a median of 27% (**A**). Examples of tumor sections with a CAIX positive fraction of 3% and 81% are depicted. The labeling index of Ki-67 varied from 0 – 42% with a median value of 14% (**B**), with a representative tissue section of 3% and 33%.

After immunohistochemical processing, Ki-67 and CAIX gave a clear brown staining with intensities varying gradually from light brown to dark brown. Clear membranous CAIX staining was observed in most tissue sections. The frequency distribution of the CAIX fraction and the Ki-67 labelling index for all tumors as obtained by automated analysis is shown in Figure 2. The CAIX positive area showed a range of 0-93% with a median value of 27%. The Ki-67 labelling index varied from 0-42% (median 14%).

To validate the automated analysis, the results were compared with visual scoring for both parameters. The area positive for CAIX showed a strong interobserver correlation (r_s = 0.96, p = < 0.0001, Figure 3a). Likewise, the correlation between the observers and the

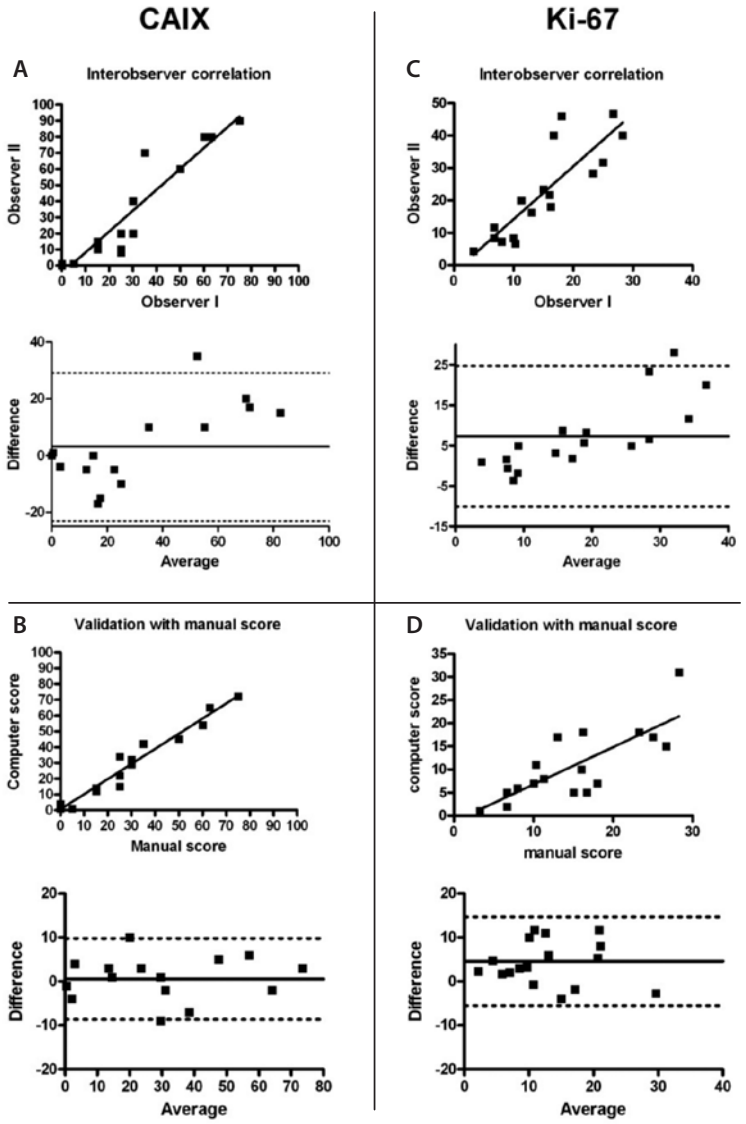

Fig. 3 Correlation of automated analysis with manual scoring. A strong relationship between CAIX score of two observers ($r_s = 0.96$, $p < 0.0001$) (**A**) and between automated measurement and manual score ($r_s = 0.97$, $p < 0.0001$) (**B**) was observed, with a small bias of -0.6 as determined with the Bland-Altman analysis. For Ki-67, the interobserver correlation was less strong ($r_s = 0.90$, $p = 0.0001$) (**C**) and the correlation with the automated analysis showed a bias of 4.2, with higher manual scores (**D**). Dashed horizontal lines represent the mean difference and the mean difference plus and minus 1.96 times the SD of the differences.

automated analysis was strong ($r_s = 0.97$, $p < 0.0001$ and $r_s = 0.93$ $p < 0.0001$) and is shown for one of the observers (Figure 3b). Bland-Altman analysis confirmed this correlation with a bias of only 0.5. For Ki-67 the interobserver correlation was less strong, but also significant ($r_s = 0.90$, $p = 0.0001$, Figure 3c). Again, the correlation with the automated analysis was significant for both observers and is shown for one of them. By using Bland-Altman analysis a bias towards higher scores by manual assessment was observed (Figure 3d).

From 15 patients two biopsies from the same tumor were available for analysis. To get an indication of the intratumor variation of CAIX and Ki-67, sections from both biopsies were analyzed. The CAIX-positive area and the Ki-67 labelling index both showed a strong correlation ($r_s = 0.81$, $p = < 0.0003$ and $r_s = 0.89$, $p = < 0.0001$, Figure 4a and b).

The effect of the grid (square) size on the results was evaluated, by using different square sizes, varying between 10 x 10, 20 x 20 and 40 x 40 pixels. For CAIX, a course grid resulted in higher values and a fine grid in lower values, as can be expected. Beforehand, a grid size of 20 pixels was chosen as the preferred size for the analysis, based on average cell size and small inaccuracies in the matching of the Ki-67 and CAIX sections. On top of that, the results obtained with this grid size had the best fit with the absolute values found by the visual scoring of the CAIX area (slope 0.99 (20) slope 0.75 (10) and slope 1.15 (40).

For the labelling index of Ki-67, varying the grid size from 20 to 10 or 40 showed little effect on the results (slopes 0.64, 0.59 and 0.62 respectively), suggesting that the outcome is independent of the size of the square compartments.

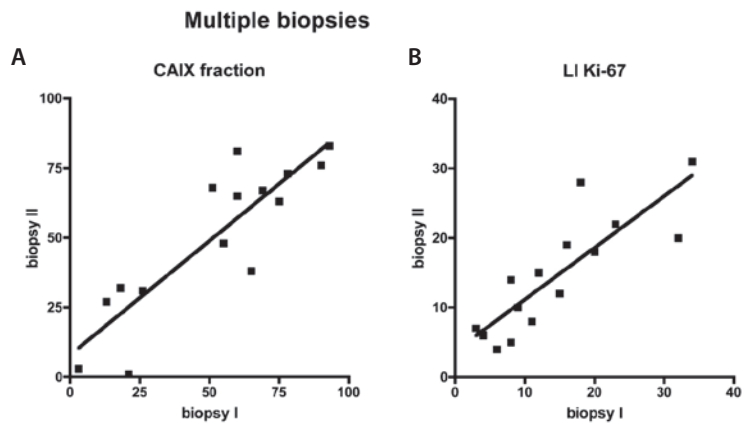

Fig. 4 Correlation of CAIX positive area and labeling index of Ki-67 in multiple biopsies of the same tumor. Both CAIX (A) and Ki-67 (B) showed a strong correlation (rs = 0.81, $p < 0.0003$ and rs = 0.89, $p < 0.0001$ respectively).

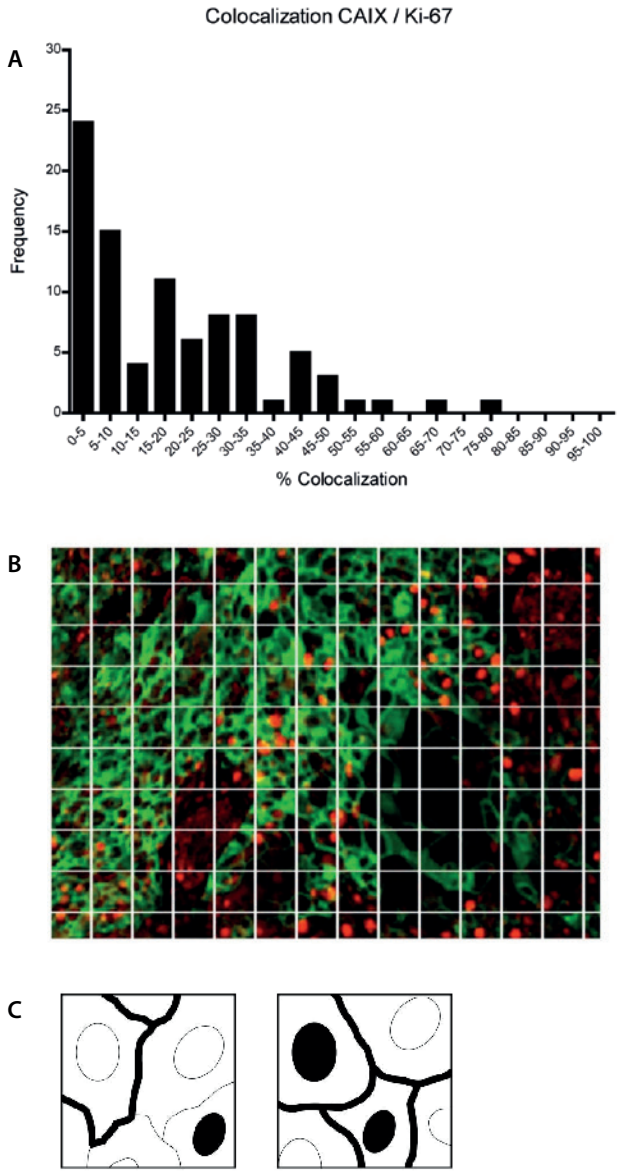

Fig. 5 Colocalization of CAIX expression and proliferation (labeling index of Ki-67) in biopsies of 89 laryngeal tumors assessed by parametric mapping. The frequency distribution (**A**) shows a wide range of colocalization (0 – 76 %), with an uneven distribution towards lower values. A peudo-coloured merged image with an overlay grid illustrates our definition of colocalization in this study (**B**). (**C**) Schematic representation of mismatch (left) and "true" colocalization (right) within a square.

As a measure for colocalization the percentage of squares with a value greater than zero for both the CAIX parameter map and the Ki-67 labelling index map was calculated for each biopsy by juxtaposition of the image maps. A wide range of colocalization was observed (0-76%), with a median value of 15% (Figure 5). The percentage of true colocalization divided by the total colocalization as calculated from the automated analysis was 79%, 82% and 84% for the 3 sections scored. The percentage of mismatch (defined by expression of both CAIX and Ki-67 within the same square, but not in the same cell, figure 5C) was 5%, 10% and 8% respectively. The remaining squares showed a discordant result due to thresholding differences between visual scoring and the preset cut off value for the automated analysis.

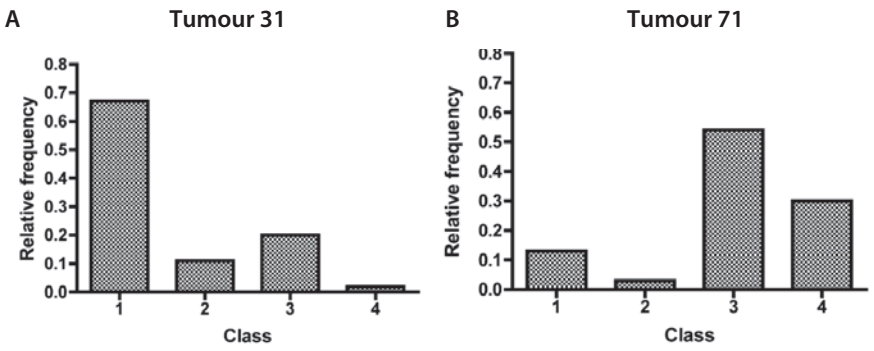

Fig. 6 Example of two tumors with a different degree of colocalization. The squares of a whole tissue section were subdivided into four classes: class 1 contains squares with CAIX intensity < 100 and Ki-67 labelling index < 25, class 2 CAIX < 100 and Ki-67 ≥ 25, class 3 CAIX ≥ 100 and Ki-67 < 25, class 4 CAIX ≥ 100 and Ki-67 ≥ 25. The relative frequency distribution of tumor 66 (**A**) shows more class 2 square compartments, tumor 71-II (**B**) more class 3, representing a different degree of colocalization.

Two examples with a comparable percentage of overall colocalization (28% and 38%), but an opposing class distribution, taking into account the intensity of CAIX staining and the value of the Ki-67 labelling index, are depicted in Figure 6. In tumor 66 the colocalization consists mainly of areas with low CAIX intensity and high Ki-67 labelling index (class 2). In tumor 71-II the class distribution is totally different with 70% of the colocalization consisting of areas with high CAIX intensity and low Ki-67 labelling index (class 3).

Discussion

Recognizing specific tumor markers that can predict outcome or response to treatment can be of great value in therapy decisions in many different types of cancer. Various single markers have already shown prognostic value, but as the aggressiveness of a tumor is a complex interaction of different pathways, knowledge about colocalization of multiple markers could be of great importance.

In this report we describe an automated computer-assisted analysis of immunohistochemically stained tumor sections using parametric mapping for simultaneous quantification of Ki-67, a nuclear proliferation marker, and CAIX, a membrane-bound hypoxia-related marker. We assessed the staining percentages of CAIX and Ki-67 individually and the percentage area showing colocalization of the two markers. To our knowledge, this is the first study that uses the parametric mapping technique to compare two biologically connected features located at different subcellular compartments.

This parametric mapping method has two strengths. The first is its potential to determine the relative tumor area positive for a membrane-bound protein in an accurate way, correlating strongly with manual assessment. The second is the measurement of colocalization of markers with different intracellular locations for a whole tumor section, which is almost impossible to assess by visual scoring. Although we focused on CAIX and Ki-67, this method is applicable to many other tumor markers, for example epidermal growth factor receptor (EGFR) and Ki-67 or hypoxia-inducible factor 1α (HIF-1α) and CAIX, and can therefore be of great significance for future research.

As our parametric mapping technique requires grayscale images as input, we first had to separate the individual colours in the sections. In this study, the resulting images obtained by the linear unmixing algorithm were of good quality and, despite a high background staining, apt for further analysis. CAIX showed a strong membranous staining, of which the cut-off value could easily be set against the background. The correlation of the automated results for CAIX and Ki-67 with manual scoring was high. No systemic errors were shown by the Bland-Altman analysis, making the parametric mapping technique an accurate method for assessing the relative CAIX-positive tumor area in whole tissue sections. Although the results with varying grid sizes all showed a strong correlation with visual scores, the linear regression slope of the 20 x 20 pixel square was closest to 1. Therefore, in absolute values this was the best estimation compared to manual scoring.

This is a somewhat unexpected finding, as theoretically the ideal square size would contain one cell. However, due to heterogeneity in cell size and tissue structure this ideal size is difficult to determine. Considering the other goal of our research: assessing the overlap of the two markers in consecutive tissue sections, the 10 x 10 pixel square would likely be too small. In conclusion, the 20 x 20 pixel size is suitable for analysis of the relative positive area of a membrane-bound marker.

Although the colocalization of CAIX and Ki-67 cannot be assessed exactly on a cellular level, this is a very close approximation. For the analysis of the colocalization the size of the square compartments of 20 x 20 pixels seemed appropriate. This size includes at least one cell and can correct for small errors in the fitting of consecutive sections, without the loss of information. As the deviations in fitting were very small and because of the regional pattern of CAIX staining[23], this is a decent and viable approach to assess the degree of colocalization. This is supported by the finding that only 5-10% mismatch is present, when observing the colocalization at the level of the square compartments.

As becomes clear in Figure 1, information about intensity values of CAIX and labelling index of Ki-67 remains present in the parameter image maps. This information was used to create classes of colocalization, with class 4 representing the strongest colocalization with intense CAIX staining and a high Ki-67 labelling index. This is illustrated in Figure 6 with the relative frequency distribution of these classes of two different tumors. Tumor 66 and tumor 71-II have similar overall colocalization rates but show a different distribution of colocalization over the various classes, possibly representing dissimilar biological behaviour. This is just an example of how the information in the image maps can be used. For other parameters alternative calculations and comparisons can be done with numerous possibilities.

Segmentation of the Ki-67 signal was sometimes difficult due to the gradual intensity scale and was subject to interpersonal interpretation. The large interobserver variation in the manual scores of the labelling index of Ki-67 confirmed this. Other authors have encountered this problem as well.[24,25] This could possibly be improved by using a different staining method, such as immunofluorescence, or altered background stain. Another issue is the higher overall labelling index for Ki-67 obtained with manual scoring compared to the automated analysis. This is probably due to the intensity scale of the DAB staining and the difference of manually counting positive nuclei on the microscope with a 400 x magnification and setting a threshold on a tissue image scan. In the first situation the estimated cut off value will be lower, resulting in a higher value. However, most importantly, the relative scoring of the observers and the automated analysis correlated well, indicating that with different observers as well as with the automated analysis tumors are ranked similarly.

As compared to a high throughput method such as tissue microarray, our parametric mapping technique is a labour-intensive method due to the delineation of the tumor areas and the matching of the tissue sections. However, a large disadvantage of tissue microarrays is the use of small tissue cores, leaving the heterogeneity within a tissue section out of account and increasing the risk of sampling error. Analysis of whole tissue sections is a major advantage of the current method. This is supported by the strong correlation seen between CAIX fractions in multiple biopsies from the same tumor. As described before, there can be large intratumor heterogeneity with respect to CAIX fraction[12] and Ki-67 labelling index[26]. The wide range we observed in CAIX staining (0 – 93

%) can be another explanation for this remarkable finding. A good correlation is more likely to occur in a wide range of values, than with small intertumor variations. For the Ki-67 labelling index a strong correlation between biopsies was shown as well, although the data showed a smaller, but still considerable range (0 – 42 %). Therefore, it can be concluded that if the range of observed values is sufficiently wide, a random biopsy could give a fairly representative indication of the hypoxic or proliferation status of a tumor. A marker with little variability would be less informative anyway as it has less potential to discriminate between tumors with different biological behaviour.

In conclusion, research on verification of specific tumor markers runs parallel to the development of targeted therapies. This enables a more elaborate prediction of tumor response and the selection of patients for a specific therapy. Automated quantification of multiple markers in immunohistochemically stained tumor sections can be of great use to achieve this goal. By parametric mapping image maps are created for each marker containing numerical data representing a particular biological feature (area, intensity, labelling index) quantitatively. Once these image maps are prepared for analysis and a threshold is set, multiple analyses can be performed, depending on the research question. Moreover, when using multiple markers, the relationship between the corresponding biological features can be studied quantitatively independent of their subcellular localization, even in adjacent tumor sections. This parametric mapping technique can have a wide application in cancer research and patient selection.

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4

Metabolic markers in relation to hypoxia; staining patterns and colocalization of pimonidazole, HIF-1α, CAIX, LDH-5, GLUT-1, MCT1 and MCT4

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Abstract

Purpose

The cellular response of malignant tumors to hypoxia is diverse. Several important endogenous metabolic markers are upregulated under hypoxic conditions. We examined the staining patterns and co-expression of HIF-1α, CAIX, LDH-5, GLUT-1, MCT1 and MCT4 with the exogenous hypoxic cell marker pimonidazole and the association of marker expression with clinicopathological characteristics.

Methods

20 biopsies of advanced head and neck carcinomas were immunohistochemically stained and analyzed. All patients were given the hypoxia marker pimonidazole intravenously 2 h prior to biopsy taking. The tumor area positive for each marker, the colocalization of the different markers and the distribution of the markers in relation to the blood vessels were assessed by semiautomatic quantitative analysis.

Results

MCT1 staining was present in hypoxic (pimonidazole stained) as well as non-hypoxic areas in almost equal amounts. MCT1 expression showed a significant overall correlation ($r = 0.75$, $p < 0.001$) and strong spatial relationship with CAIX. LDH-5 showed the strongest correlation with pimonidazole ($r = 0.66$, $p = 0.002$). MCT4 and GLUT-1 demonstrated a typical diffusion-limited hypoxic pattern and showed a high degree of colocalization. Both MCT4 and CAIX showed a higher expression in the primary tumor in node positive patients ($p = 0.09$ both).

Conclusion

Colocalization and staining patterns of metabolic and hypoxia-related proteins provides valuable additional information over single protein analyses and can improve the understanding of their functions and environmental influences.

Background

Malignant tumors often exhibit an altered metabolism compared to normal tissues. This phenomenon can be explained by several underlying mechanisms. First of all, the genetic changes related to a high proliferation rate, as observed in many tumors, lead to an increased metabolism.[1] Another important reason for a changed metabolism is the adaptation of tumor cells to the microenvironment. Due to rapid tumor growth, hypoxic areas are frequently encountered. Under circumstances of severe hypoxia, cells are forced to use anaerobic glycolysis as their primary energy source, the Pasteur effect.[2] Normal cells convert to oxidative phosphorylation when oxygen levels are restored. In contrast, tumor cells can use aerobic glycolysis even in the presence of sufficient amounts of oxygen. This is called the Warburg effect, a manifestation of a modification of the tumor cell metabolism.[3] Due to a high level of aerobic glycolysis, in many tumor cells, glucose consumption is substantially higher than in normal cells.[4,5]

The consequence of the high rate of glycolysis in malignant cells is the production of large amounts of lactic acid. An interesting observation made by Sonveaux et al. is the preference of tumor cells for lactic acid over glucose as the primary energy source.[6] This creates the perfect conditions for a symbiosis between anaerobic glycolytic cells and aerobic tumor cells[6] or aerobic stromal cells, as described in colorectal carcinomas.[7]

Recently, monocarboxylate transporters (MCT's) have been discovered to play an important role in this symbiosis. These transporters facilitate the uptake and excretion of monocarboxylates, like lactate and pyruvate, and act as monocarboxylate-proton symporters.[8] MCT4 is a low-affinity / high capacity lactate transporter, which is abundantly present in highly glycolytic muscle cells. It is one of the many target genes of hypoxia-inducible factor 1 (HIF-1).[9] MCT1 is a high-affinity, low capacity monocarboxylate transporter, found in normal tissues like the intestinal epithelium (executing an important role in organic acid absorption), the blood brain barrier, red blood cells and skeletal muscle cells. Its expression seems to be regulated by multiple signaling pathways, microenvironmental parameters, changes in substrate concentration and pH.[8] Other important proteins related to the metabolism of tumor cells are glucose transporter-1 (GLUT-1), the main transporter involved in glucose influx, and lactate dehydrogenase-5 (LDH-5), responsible for the conversion of pyruvate into lactate. Like MCT4, these proteins are upregulated under hypoxic conditions by HIF-1.[10] Another main target for HIF-1 is carbonic anhydrase IX (CAIX), a hypoxia-related protein involved in pH regulation[11], that shows weak correlations with the exogenous hypoxia marker pimonidazole.[12,13] The advantage of the use of these proteins as endogenous immunohistochemical markers is that no prior infusion of markers is necessary and therefore archived material can be used to assess the metabolic and, possibly, the hypoxic status of the tumor. However, up until now no endogenous marker has been identified that correlates strongly with pimonidazole.[14] In this study, we describe and quantify the expression patterns and colocalization of several important hypoxia-

related and metabolic markers in biopsies of head and neck tumors and in particular the association with pimonidazole as the reference exogenous hypoxic marker.[15]

Methods

Samples

The study was approved by the local ethics committee. 20 biopsies from 18 head and neck tumors were included in the analysis; from two tumors two biopsies were available. All patients received pimonidazole (1-((2-hydroxy-3-piperidinyl)propyl)-2-nitroimidazole hydrochloride, Hypoxyprobe-1; Natural Pharmacia International, Belmont, MA) intravenously (500mg/m^2) two hours before biopsy taking. Pimonidazole is a bioreductive chemical probe that forms protein adducts in viable hypoxic cells. Biopsies were snap frozen in liquid nitrogen and stored until further processing. The samples were cut in sections of 5µm and stained by immunofluorescence for pimonidazole, HIF-1α, CAIX, GLUT-1, LDH-5, MCT1, MCT4 and vessels in different combinations of 3 markers per tissue section.

Immunohistochemistry

For immunohistochemical processing, the sections were fixed for 10 minutes in acetone and rehydrated in PBS 0.1 mol/L (pH 7.4) (Klinipath, Duiven, The Netherlands). Between all consecutive steps of the staining procedure the sections were rinsed thrice for 5 minutes in PBS.

For detection of pimonidazole, sections were incubated with rabbit-anti-pimo antibody (J.A. Raleigh, Department of Radiation Oncology and Toxicology, University of North Carolina, Chapel Hill, North Carolina, USA) diluted 1:1000 in primary antibody diluent (PAD, Abcam, Cambridge, UK) for 30 minutes at 37°C. The second incubation step was with donkey-anti-rabbit Alexa488 (Molecular Probes, Leiden, The Netherlands) diluted 1:600 in PBS. Staining for vessels was done by incubation with the mouse antibody PAL-E (Euro Diagnostica, Arnhem, The Netherlands) diluted 1:10 in PAD, followed by incubation with chicken-anti-mouse Alexa647 (Molecular Probes) for 60 min at 37°C diluted 1:100 in PBS.

The same secondary antibody (in a different tissue section) was used to detect CAIX, after incubation with mouse-anti-CAIX antibody (E. Oosterwijk, Department of Urology, University Medical Center, Nijmegen), diluted 1:25 in PAD for 30 min at 37°C.

For detection of MCT1, sections were incubated with goat-anti-MCT1 (Santa Cruz)[7], 1:100 in PAD, overnight at 4°C. The next day the secondary antibody was added: donkey-anti-goatCy3 (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) 1:600 in PBS, 30 min at 37°C.

Staining for MCT4 was done by incubation with rabbit-anti-MCT4 (Santa Cruz)[16], 1:100 in PAD, overnight at 4°C. The secondary antibody was either goat-anti-rabbitFabCy3 (1:600 in PBS) or donkey-anti-rabbitAlexa488 (1:600 in PBS) depending on the combination of markers stained in that tissue section.

Sections were incubated with rabbit-anti-GLUT-1 (Neomarkers, Fremont, CA, USA.), diluted 1:100 in PAD for 30 min at 37°C, followed by donkey-anti-rabbitAlexa488, 1:600 in PBS for 30 min at 37°C for detection of GLUT-1.

Staining for HIF-1α was done by incubation with rabbit-anti-HIF-1α (Santa Cruz), diluted 1:50 in PAD overnight at 4°C, followed by goat-anti-rabbitCy3 (Jackson Immuno Research Laboratories Inc.), 1:600 in PBS for 30 min at 37°C. In the same section, all nuclei were stained with Hoechst (Sigma, Zwijndrecht), 0.33 µg/ml, in PBS for 5 min at room temperature.

Finally, for detection of LDH-5 sections were incubated with sheep-anti-LDH-5 (Abcam), diluted 1:100 in PAD overnight at 4°C. The next day, incubation with donkey-anti-sheepCy3 (Jackson Immuno Research Laboratories Inc.), 1:600 in PBS for 30 min at 37°C, completed the staining.

All sections were mounted in fluorostab (ProGen Biotechnik GmbH, Heidelberg, Germany).

Image acquisition and analysis

The tissue sections were scanned using a digital image processing system consisting of a high-resolution 12-bit CCD camera (Micromax, Roper Scientific Inc., Trenton, NJ, USA) on a fluorescence microscope (Axioskop, Zeiss, Göttingen, Germany) and a computer-controlled motorised stepping stage. Image processing was done using IPLab software (Scanalytics Inc., Fairfax, VA, USA) on a Macintosh computer.[17] Each tissue section was sequentially scanned for the three signals at 100x magnification with a resolution of 2.6 µm / pixel. The resulting composite grey scale images were converted to binary images for further analysis. Thresholds for the fluorescent signals were interactively set above the background for each individual marker. The composite grey scale images were superimposed into one pseudocolored image for visual evaluation.

Guided by an H&E stained consecutive section, the tumor area of each section was delineated. This area was subsequently used as a mask in further analysis from which non-tumor tissue, large necrotic areas and artefacts were excluded. The marker fractions were defined as the tumor area positive for the marker, divided by the total tumor area. With this method, an automated quantitative analysis of the percentage of positively stained tumor tissue can be obtained. To determine the colocalization of the various markers with pimonidazole, the relative area positive within and outside the pimonidazole stained area was calculated. Similar analyses were done to assess the colocalization between MCT1, MCT4, GLUT-1 and CAIX.

The spatial distribution of the markers in relation to the blood vessels was measured by calculating the relative area positive in six zones around the closest vessels with a width

of 50 µm each (0-50µm, 50-100µm, 100-150µm, 150-200µm, 200-250µm and>250µm).[17] As the absolute fractions differ greatly, normalisation of fractions was performed for clear comparison of distributions. Quantitative analysis of the HIF-1α staining was not possible because of the low signal-to-background ratio in many HIF-1α-positive areas. All sections were visually scored by two observers and divided in two groups; high and low HIF-1α expression based on the intensity and the estimated fraction of HIF-1α positive cells. Differences between the observers were resolved at the microscope.

Statistics

Statistical analyses were done with Prism software package for Macintosh. The relationships between metabolic parameters (as continuous variables) were tested using the Spearman rank correlation coefficient or the Pearson correlation coefficient as appropriate. Differences in colocalization were analyzed with a Mann-Whitney test. To test the association of the markers with categorical tumor characteristics (T-stage, N-stage and histopathological grade) either the Mann-Whitney test or Fisher's exact test (after dichotomization of the variables) was used.

Results

20 biopsies of 18 patients with histologically confirmed advanced stage squamous cell carcinoma of the head and neck were included in this study. Tumor characteristics are shown in Table 1.

Correlation between metabolic markers and tumor characteristics

A trend to a higher expression of both MCT4 and CAIX in node positive patients was observed (p = 0.09 both) (Figure 1). After dichotomization of the variables MCT4 expression was significantly higher in node positive patients (p = 0.01). HIF-1α only showed a trend towards higher expression in node positive patients (p = 0.06), although HIF-1α expression was only found in one node negative tumor. In this biopsy the HIF-1α expression as well as the pimonidazole staining was found in well-differentiated areas around keratinization. T4 tumors showed a significantly lower pimonidazole staining (p = 0.02) and CAIX expression (p = 0.03) than T2 or T3 tumors. This inverse trend was found for MCT1 and MCT4 as well (p = 0.09 and p = 0.08 respectively). No correlations were found between any of the markers and differentiation grade.

Staining patterns

Examples of staining of all markers are shown in Figure 2. Pimonidazole fraction ranged from 0 - 34% (median 7%) and different hypoxic patterns were observed. Some tumors showed a typical ribbon-like pattern, others a more patchy pattern.[18] (Figure 2A and B)

Table 1 Tumour site, stage and grade

Characteristics	Number
Tumour site	
Larynx	7
Hypopharynx	4
Oropharynx	5
Oral cavity	2
T stage	
T2	3
T3	6
T4	9
N stage	
N0	6
N+	12
Histopathological grade	
Moderately differentiated	14
Poorly differentiated	4

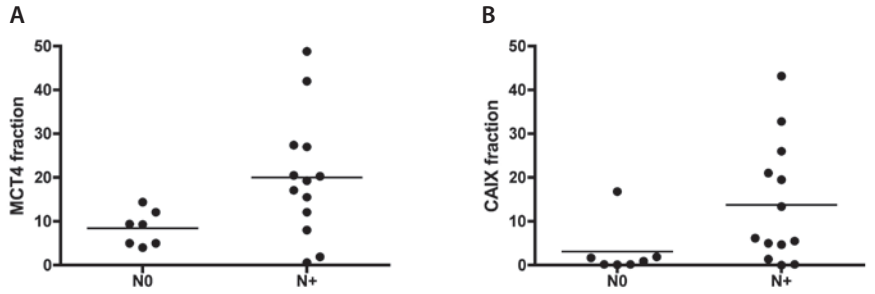

Fig. 1 A significantly higher expression of MCT4 (p=0.04, a) and CAIX (p=0.05, b) was found in node-positive tumours. One tumor without nodal metastasis had a high CAIX expression of 17%. Interestingly, no HIF-1α expression was found in this tumor.

Extensive cytoplasmic LDH-5 expression was seen in most of the tumors (fraction range 17 - 91%, median 37%, figure 2C), with more intense staining in hypoxic areas.

Membranous CAIX expression closely followed the hypoxic pattern in some tumors, in others CAIX staining showed less spatial correlation with pimonidazole. The fraction ranged from 0 - 43% (median 4.7%) (Figure 2A, B and D).

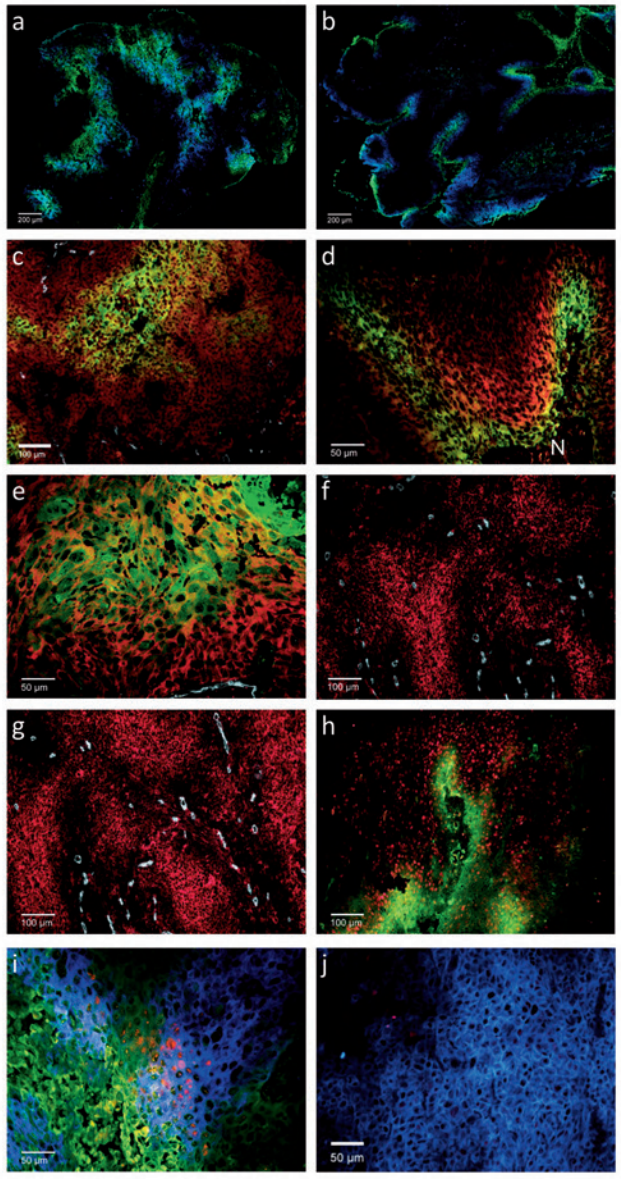

Fig. 2 (**A** and **B**) Fluorescent microscopic images of two head and neck carcinomas with different pimonidazole (green) and CAIX (blue) staining patterns.
(**C** – **H**) Examples of staining of the various markers. (**C**) LDH-5 (red), pimonidazole (green) and vessels (white). (**D**) Perinecrotic pimonidazole (green) and CAIX (red) staining, N = Necrosis. (**E**) MCT1 (red), pimonidazole (green) and vessels (white), note the co-staining of MCT1 with pimonidazole. (**F** and **G**) Membranous GLUT-1 (**F**) and MCT4 (**G**) (both red) staining in relation to the vessels (white). (**H**) Nuclear HIF-1α (red) expression in relation to pimoniazole (green) staining. (**I** and **J**) Fluorescent microscopic images showing intense HIF-1α (red) staining in a pimonidazole-related (green) CAIX (blue) area and almost absent HIF-1α staining in another CAIX area in the same tissue section.

MCT 1 showed a clear membranous staining pattern with substantial variation in intensity and extent; in some sections large MCT1 positive areas were seen, in other sections almost no MCT1 was present (fraction range 0.1 - 64%, median 14%). MCT1 staining was not only observed adjacent to hypoxic areas, but also within hypoxic areas (Figure 2E).

MCT4 showed a more diffuse staining pattern, with fractions varying from 0.6 - 49% (median 14%) and with more staining at increasing distance from the vessels. GLUT-1 had a comparable staining pattern to MCT4 (fraction range 3 - 43%, median 18%): diffuse staining throughout the tissue section, with more staining at increasing distance from the vessels. (Figure 2F and G) Apparent localised nuclear HIF-1α staining was present in 15 out of 20 biopsies, mostly in and around hypoxic areas (Figure 2H) with large variation in intensity. In some tissue sections membranous or cytoplasmatic staining was present as well, but only nuclear staining was taken into account.

Marker	Pimonidazole r_s (p)		CAIX r_s (p)		MCT1 r_p (p)		MCT4 r_p (p)		GLUT-1 r_p (p)	
LDH-5	0.66	**p=0.002**	0.35	p=0.13	0.09	p=0.71	0.27	p=0.23	0.39	p=0.09
GLUT-1	0.16	p=0.51	0.68	**p=0.001**	0.36	p=0.12	0.58	**p=0.008**		
MCT4	0.21	p=0.37	0.75	**p<0.001**	0.18	p=0.46				
MCT1	0.16	p=0.51	0.62	**p=0.003**						
CAIX	0.42	p=0.06								

Marker	HIF-1α +	HIF-1α -	P-value
CAIX	17%	3%	0.02
MCT4	22%	10%	0.05
GLUT-1	24%	14%	0.06
LDH-5	23%	13%	0.14
MCT1	49%	44%	0.58
Pimonidazole	11%	9%	0.91

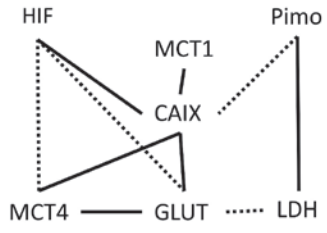

Fig. 3 Correlation between the six endogenous markers and pimonidazole with a schematic representation. Solid lines represent a significant correlation, dashed lines a trend (p-value between 0.05-0.1).

Correlations between markers

To get a global impression of the associations between the six markers overall correlations were calculated, irrespective of the geographical distribution. (Figure 3) The strongest correlation with pimonidazole was observed for LDH-5 fraction ($r = 0.66$, $p = 0.002$). CAIX showed significant correlations with MCT1, MCT4 and GLUT-1.

The expression of the endogenous markers was compared between biopsies with a high and low HIF-1α expression. (Figure 3) Overall, biopsies with a high HIF-1α expression demonstrated a significantly higher CAIX fraction. Remarkably, two biopsies clearly showed HIF-1α expression, but very low fractions of CAIX, GLUT-1 and MCT4. In one of the biopsies pimonidazole staining was associated with well-differentiated keratinizing areas as described by Janssen et al.,[19] the other biopsy showed no pimonidazole staining at all. Overall, mean pimonidazole staining was equal in biopsies with high and low HIF-1α expression. A schematic representation of the associations between the markers is shown in Figure 3.

Colocalization of metabolic and hypoxic markers

The results of the quantitative analysis of the colocalization of the various markers are shown in Figure 4. MCT4 expression was significantly higher in hypoxic areas than in non-hypoxic areas ($p = 0.001$) and also clearly correlated to CAIX expression. GLUT-1 expression and MCT4 expression showed an even stronger amount of colocalization with approximately six times higher GLUT-1 expression in MCT4 positive areas.

MCT1 was present in well-oxygenated areas, but unexpectedly showed considerable expression in hypoxic areas as well (no significant difference, $p = 0.11$). Additionally, MCT1 expression was significantly correlated with CAIX expression ($p < 0.001$). Pimonidazole stained areas exhibited, beside MCT4, a significantly higher expression of CAIX and LDH-5 compared to pimonidazole negative areas.

Although colocalization with HIF-1α expression could not be analyzed in the same manner as the other markers, it was observed that pimonidazole-related CAIX areas showed more intense HIF-1α expression than CAIX areas far from pimonidazole positive hypoxic areas (Figure 2 I and J).

Relationship of markers with vasculature

Chronic hypoxia is an important feature of malignant tumors, marked by a tissue oxygen gradient with lower oxygen tensions farther from the blood vessels. The expression of the endogenous markers in relation to the vessels, with pimonidazole as a reference hypoxic marker, can provide valuable information about their usefulness as a marker of chronic hypoxia. To assess the expression of the markers in relation to the vessels, fractions were calculated at different distances from the most nearby vessel in steps of 50 µm. In general, hypoxic fraction and LDH-5 staining increased at larger distances from the vessels. GLUT-1 and MCT4 were present in variable fractions in the proximity of vessels, but the expression

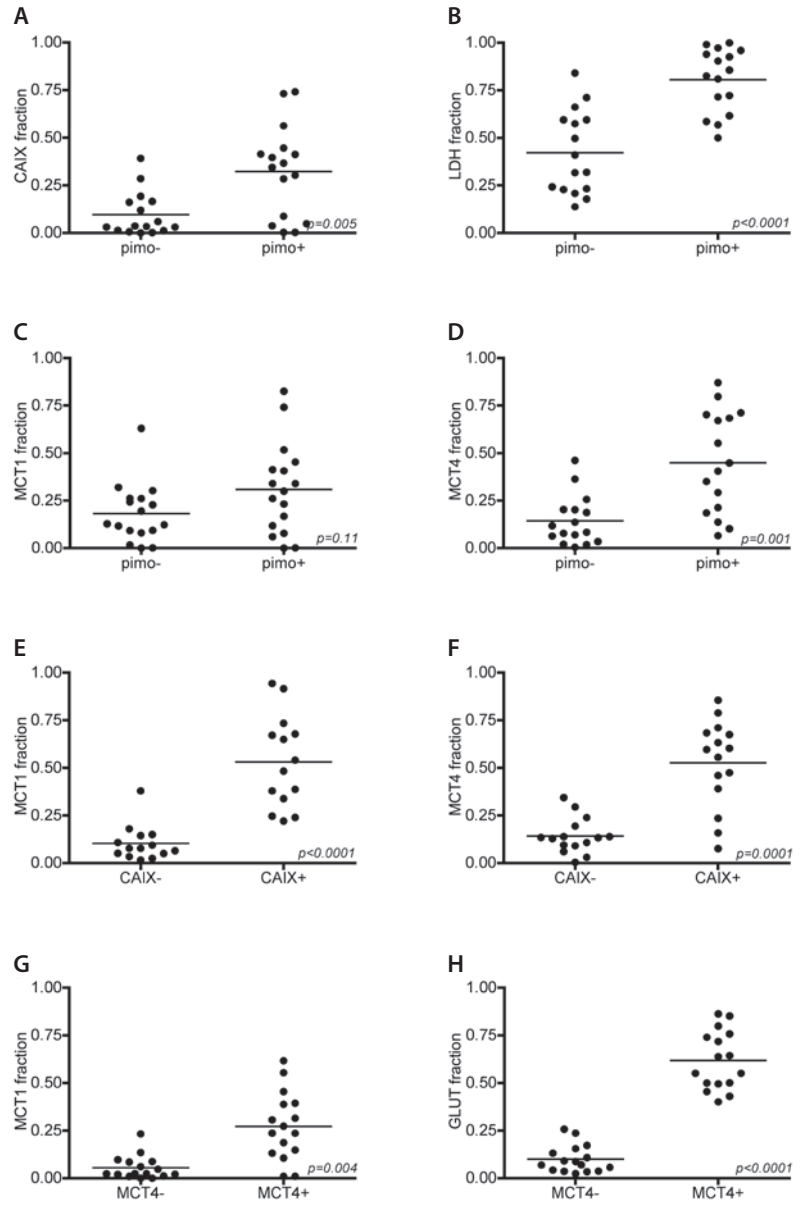

Fig. 4 Colocalization of the various markers. (**A**-**D**) CAIX, LDH and MCT4 show a significantly higher expression in pimonidazole stained areas, MCT1 expression is not significantly different. (**E** and **F**) Expression of MCT1 and MCT4 in CAIX negative and positive areas, both are significantly higher in areas expressing CAIX. (**G** and **H**) Increased expression of MCT1 and GLUT in MCT4 positive areas.

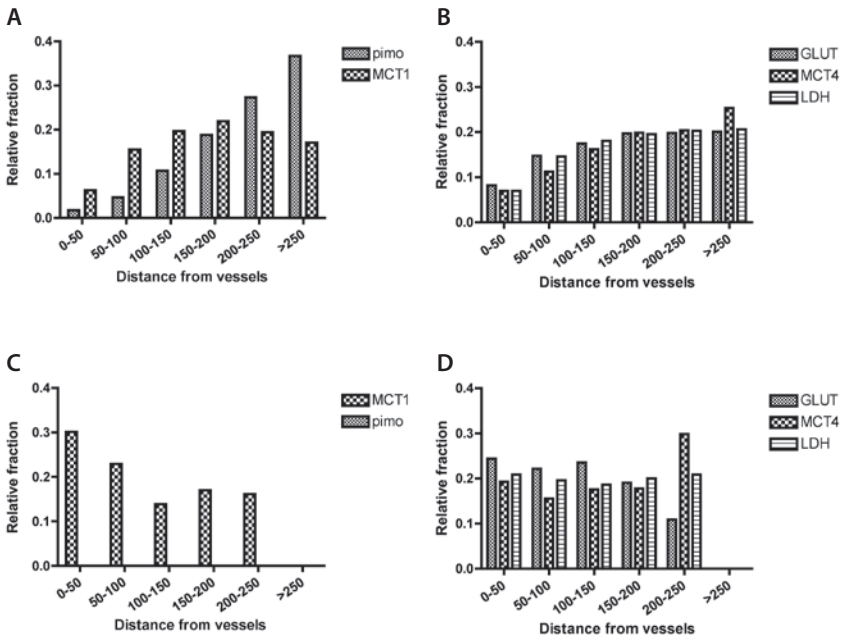

Fig. 5 Marker expression in different zones around the vessels in a biopsy with a large hypoxic fraction (upper panels) and a tumor with no pimonidazole staining (lower panels). Pimonidazole staining increases steadily at larger distances from the vessels (**A**). The increase of GLUT, MCT4 and LDH expression is less steep with a plateau reached between 150-200 mm (**B**). MCT1 expression increases until 150-200 mm, a slight decrease is noted at larger distances (**A**). In the tumour with no pimonidazole staining, no increase of GLUT, MCT4 and LDH expression is observed (**D**) and a decrease in MCT1 expression at larger distances from the vessels (**C**).

increased about two to four-fold at > 150 µm from the vessels. All except four tumors exhibited this "chronic hypoxic pattern". These remaining four showed no pimonidazole staining. Two examples of the distribution of the markers in tumors with pimonidazole (chronic hypoxic pattern) and without pimonidazole staining (non-hypoxic pattern) are shown in Figure 5. In one tumor (5a and b) with a large hypoxic fraction (20%) pimonidazole binding increases steeply with distance from the vessels. GLUT-1, MCT4 and LDH-5 follow this pattern but less steep, with a plateau reached between 150-200 mm. MCT1 expression increases until 150-200 mm and decreases at larger distances. In the second tumor (Figure 5c and d) all metabolic markers were strongly expressed without any relationship with the vessels and, in the absence of pimonidazole, not depending on hypoxia. Low HIF-1α and CAIX expression was present in this tumor.

MCT1 expression clearly showed a different pattern compared to the other markers; in most tumors the MCT1 fraction increased slightly till 100 µm from the vessels and remained constant or modestly decreased again at larger distances. However, in the four tumors without hypoxia, MCT1 expression was present close to the vessels and decreased farther from the vessels.

Discussion

Hypoxia is an important feature of advanced head and neck tumors, with a negative influence on prognosis.[12,20] Evaluation of cellular responses to hypoxia can be of clinical relevance in a prognostic and predictive way and possibly for treatment adaptation. Until now no endogenous marker has been found that strongly and consistently correlates with hypoxia, so different kind of analyses as presented here are crucial to elucidate the role of the various proteins and the response of tumor cells to a hypoxic microenvironment.

Although a global analysis of the overall expression of a certain protein allows easy comparison between biopsies and between different studies, it is a huge simplification of the true heterogeneous situation in a tumor biopsy. Therefore, to gain more insight in the spatial relationship between the metabolic markers and the oxygenation status in the tumor, we additionally assessed the expression of the proteins within and outside pimonidazole stained areas and the relation to the vessels. With this analysis more markers show an evident relationship with pimonidazole than with a global correlation analysis (Figure 4).

Of the endogenous hypoxia-related markers that we examined, LDH-5 has the strongest relationship with pimonidazole. LDH-5 is one of the target enzymes of HIF-1 and has been described to have a strong association with HIF-1α expression in tumor tissue sections.[21,22] The high expression of LDH-5 in hypoxic areas is as expected; it is one of the key enzymes in the glycolysis, the primary energy source in the absence of oxygen. The reason for a considerable expression of LDH-5 outside hypoxic areas could be the high rate of aerobe glycolysis (the Warburg effect) as often observed in malignant tumors[4], although alternative explanations are possible, as pyruvate can originate from other pathways as well[23]. It should be noted that due to the similarity between LDH-5 and the other LDH isoforms, there is a possibility of some cross-reactivity of the antibody.

CAIX is previously described to correlate weakly with pimonidazole binding.[13,24,25] Despite the weak overall correlation, CAIX fraction was significantly higher in the hypoxic (pimonidazole positive) areas. This emphasizes the strong association of this protein with hypoxia that could not be found by a simple correlation analysis. Interestingly, although all the endogenous markers, with the exception of MCT1, are regulated by HIF-1[9,10], the staining patterns and colocalization differ. An interesting observation is the strong resemblance of the HIF-1α and CAIX staining pattern (intermediate hypoxic areas). This

typical pattern circumferences the pimonidazole stained area with a partial overlap (Fig. 2I). The colocalization of CAIX with HIF-1α is far from perfect, but more concordant than that of GLUT-1, MCT4 and LDH-5 with HIF-1α The strong relationship of CAIX with HIF-1 is known, with CAIX expression tightly controlled by HIF-1.[26] In this context, an interesting observation is the higher level of HIF-1α expression in hypoxia-related CAIX areas than in CAIX areas separate from hypoxic (pimonidazole stained) areas (Figure 2I and J). A possible explanation of these findings could be the increased transcription of HIF-1α without stabilization in the intermediate hypoxic areas (areas with CAIX expression without HIF-1α staining) and stabilization of HIF-1α in severe hypoxic areas (areas overlapping and adjacent to pimonidazole with CAIX and HIF-1α staining).[27] It could also indicate the transient presence of acute hypoxic areas, which are reoxygenated before pimonidazole administration. It was shown in SiHa (human cervical squamous cell carcinoma) tumors that up to 20% of the tumor cells were intermittently hypoxic over an 8-hour period.[28] This explanation is less likely as CAIX upregulation requires a longer period of hypoxia.[29] A third explanation could be the induction of HIF-1α and consequent activation of CAIX through a hypoxia-independent mechanism without stabilization of HIF-1α under these conditions. An acidic tumor microenvironment can influence HIF-1α and CAIX expression as well.[29,30] In any case, there is little evidence for a HIF-1 independent CAIX activating mechanism.[26]

Most markers showed a range of co-expression, but a very strong spatial relationship was found between GLUT-1 and MCT4 (Figure 4H). These showed a comparable, typical expression increasing at larger distances from the vessels, with a two- to four-fold increase of the fraction at 150-200 µm. This pattern and agreement reflects their interrelated role in the glycolytic pathway under hypoxic conditions, GLUT-1 for glucose import and MCT4 for lactate export. LDH-5, one of the intermediary enzymes in this pathway, shows a similar pattern, but was not stained in the same section as MCT4 or GLUT-1, so the amount of colocalization could not be calculated.

Although a small series, a positive correlation between MCT4 and N-stage was found. Overexpression of MCT4 in malignancies has been described in colorectal cancer[31] and cervical carcinomas[32]. Except for a trend towards shorter overall survival with MCT4 positive adenosquamous carcinomas of the cervix, no correlations with clinicopathological data have been found before. These findings indicate that MCT4 is a potential marker for the aggressiveness of a tumor.

MCT1 expression in > 5% of the tumor area was present in 14 of the 20 biopsies. MCT1 expression in tumors has been described in lung cancer[33], brain tumors[34] and cervical cancer[32]. However, in the colonic epithelium, Lambert et al. found a decline in expression associated with transition to malignancy.[35] In our study, MCT1 expression was present in oxic as well as hypoxic areas, in contrast to the observation made by Sonveaux et al. in biopsies of lung carcinomas[6]. It is important to note that in our study a different antibody was used of which some aspecificity formally can not be excluded, although other studies

show good results with this antibody as well.[7,36] As the fraction MCT1 even increased at larger distance from the vessels, it seems likely that MCT1, beside MCT4, plays a role in lactate export in hypoxic areas. It is reasonable to assume that in tumor cells, like in red blood cells, depending on the substrate concentrations and pH, MCT1 functions as either a lactate importer or assists MCT4 in lactate export.[37] Either way, MCT1 has already shown some potential as a therapeutic target in vitro. Inhibition of MCT1 by lonidamine induced a strong decrease in intracellular pH and loss of viability of the tumor cells.[34] The MCT1 inhibitor α-cyano-4-hydroxycinnamate blocks lactate-fueled respiration in tumor cells and induces tumor growth retardation in a mouse model.[6] Of interest is the strong correlation and colocalization of MCT1 with CAIX (Figure 3 and 4E), affirming an important role in pH regulation as described in melanoma and neuroblastoma.[34,38]

In conclusion, metabolic markers show a strong but irregular relation with hypoxia with obvious correlations between markers, emphasizing the complex metabolic regulatory system with a strong environmental (hypoxia, pH) influence. Co-expression of markers provides additional information over single marker fractions. MCT4 and GLUT-1 show a typical "diffusion-limited hypoxic" pattern with a strong colocalization indicating activation by similar stimuli. The positive correlation with N-stage makes MCT4 a potential marker for tumor aggressiveness, but its exact value still has to be established. MCT1 overexpression is present in the majority of the advanced head and neck carcinomas in our series, in non-hypoxic as well as hypoxic areas. The strong colocalization with CAIX suggests an important role in pH regulation.

Conclusion

Endogenous metabolic and hypoxia-related markers can be of great importance as prognostic and predictive markers and are potential therapeutic targets. As the various markers respond differently to hypoxia and other environmental factors, a combination of these markers could be used to predict treatment outcome and select the appropriate patients for new targeted therapies.

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5

Pattern of CAIX expression is prognostic for outcome and predicts response to ARCON in patients with laryngeal cancer treated in a phase III randomised trial

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Abstract

Purpose
In a phase III trial in patients with advanced stage laryngeal carcinoma comparing ARCON (accelerated radiotherapy with carbogen breathing and nicotinamide) to accelerated radiotherapy alone (AR) the prognostic and predictive value of CAIX, a hypoxia-associated protein, was investigated.

Methods
261 paraffin embedded tumor biopsies and 79 fresh frozen biopsies from patients entered in the trial were immunohistochemically stained for CAIX. CAIX-fraction and CAIX expression pattern were related to tumor control and patient survival.

Results
Low CAIX-fraction was prognostic for worse regional control and overall survival in patients treated with AR. Patients with a low CAIX-fraction treated with ARCON had better regional control and metastasis-free survival compared to AR (RC 97% vs 71%, p<0.01 and MFS 92% vs 69%, p=0.06).

Patients with a perinecrotic CAIX staining pattern had a significantly worse local control, metastasis-free and overall survival compared to patients with a diffuse pattern (65% vs 84%, p=0.01, 70% vs 96%, p<0.01 and 42% vs 71%, p<0.01 respectively), and this could not be improved with ARCON. After multivariate analysis CAIX pattern and N-stage emerged as significant predictors for metastasis-free survival and overall survival.

Conclusion
ARCON improves regional control and metastasis-free survival only in patients with low CAIX expression. The different patterns of CAIX expression suggest different mechanisms of upregulation and have important prognostic value.

Introduction

Over the years, the treatment for patients with advanced laryngeal cancer has shifted from laryngectomy to organ preservation strategies such as chemoradiation and radiotherapy with altered fractionation schedules. Still, a significant proportion of patients will undergo salvage laryngectomy and lose their natural voice or die from the disease due to locoregional recurrence or distant metastases. A current interest in clinical research is to identify biological treatment resistance mechanisms with the aim to deliver more effective and less toxic treatments.

An extensively studied adverse prognostic factor in solid tumors is hypoxia. Hypoxic tumors are more resistant to therapy and have a worse outcome compared to well-oxygenated tumors.[1,2] Several exogenous and endogenous hypoxia markers have been studied to assess their value as quantitative measures of hypoxia as well as their prognostic potential.[3] Endogenous markers can be assessed retrospectively on a large scale as opposed to exogenous markers, which have to be injected intravenously before taking a tumor sample. Here we studied carbonic anhydrase IX (CAIX), one of the targets of hypoxia-inducible-factor-1 (HIF-1). CAIX is involved in the respiratory gas exchange and acid-base balance and assists in maintaining intracellular pH under hypoxic conditions.[4] In a number of studies CAIX has shown to be a prognostic marker and was associated with worse locoregional control and overall survival.[4]

To specifically target hypoxic tumors, ARCON (accelerated radiotherapy with carbogen breathing and nicotinamide) was introduced in clinical studies in the early 1990s. ARCON combines radiotherapy with carbogen breathing and nicotinamide to counteract diffusion-limited and perfusion-limited hypoxia, respectively. From 2001 to 2008 a large randomized phase III trial in the Netherlands investigated accelerated radiotherapy (AR) versus ARCON in patients with advanced carcinoma of the larynx.[5] The biopsy material from the patients in that trial has been used for the current study with the objective to assess the prognostic and predictive value of CAIX with respect to AR and ARCON.

Material and methods

Patients
In the ARCON phase III trial 345 patients with advanced laryngeal cancer were randomized between AR and ARCON from 2001 to 2008. Inclusion criteria were: histological confirmation of squamous cell carcinoma of the larynx, stage T2b-T4, all N-stages, no distant metastasis, WHO performance status 0-1, age > 18 years, and written informed consent. Radiotherapy in the experimental arm was combined with carbogen (98% O_2 and 2% CO_2) breathing and nicotinamide (60 mg/kg orally) administration 1-1.5 h before treatment.

Biopsy material obtained for routine purposes of 303 of these patients was retrieved from 37 pathology departments in the Netherlands. The biopsies had been fixed in formaldehyde and were paraffin-embedded.

Additionally, from 79 patients fresh frozen biopsies were obtained 2 h after injection of the nitroimidazole hypoxia marker pimonidazole. Seventy of these patients are included in both analyses as paraffin material was present as well.

Besides for validation of the results by different staining and analysis techniques, these frozen samples were also used to study the relation of CAIX with pimonidazole, vascular density (VD) and the relative vascular area (RVA). Frozen tissue sections are better suited for staining and analysis of multiple markers.

Immunohistochemistry

All samples were cut in sections of 5 µm. One section was stained for haematoxylin and eosin to distinguish tumor from non-tumor tissue.

Paraffin-embedded sections were immunohistochemically stained for CAIX as described earlier.[6] Mouse-anti-CAIX (E. Oosterwijk, department of Urology, Radboud University Nijmegen Medical Centre) was used as the primary antibody and biotin-labelled-F(ab')$_2$-donkey-anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was applied as the secundary antibody. A negative control without the primary antibody was included.

From the frozen biopsy material, sections of 5 µm were cut and mounted on poly-l-lysine coated slides and stored at –80 °C. For detection of CAIX, the sections were incubated with mouse-anti-CA-IX antibody (E. Oosterwijk).[7] The second incubation was with goat-anti-mouse (Fab) Cy3 antibody (Jackson Immunoresearch Laboratories) The vessels were stained by incubation with the mouse antibody PAL-E (Euro Diagnostica, Arnhem, the Netherlands), followed by incubation with chicken-anti-mouse Alexa467 antibody (Molecular Probes Inc., Eugene, OR, USA).

Image analysis

For analysis of the paraffin-embedded biopsies a digital image processing system for quantitative analysis of immunohistochemically stained sections using bright field microscopy was used, as described earlier.[6] Whole tumor sections were scanned with a 10x objective at 100x magnification using a Macintosh computer running IPLab for Macintosh (Scanalytics Inc., Fairfax, VA, USA) resulting in RGB color composite images. Haematoxylin/eosin stained sections were used as a guide to manually delineate the tumor area, excluding normal tissue, necrotic areas and artefacts, creating a mask for image analysis.

After subtraction of a background image, the individual colours from the DAB (brown) and haematoxylin (blue) signals were separated, resulting in grayscale images for each color. For further processing, a threshold for each signal was interactively set and the area positive for CAIX was calculated by parametric mapping as published previously.[6]

The frozen tissue sections were scanned at 100x magnification using a digital image processing system consisting of a high-resolution 12-bit CCD camera (Micromax, Roper Scientific Inc., Trenton, NJ, USA) on a fluorescence microscope (Axioskop, Zeiss, Göttingen, Germany) and a computer-controlled motorized stepping stage. [8] Image processing was done using IPLab software (Scanalytics Inc., Fairfax, VA, USA) on a Macintosh computer. The resulting composite grayscale images were converted to binary images for further analysis. For this conversion, thresholds for the fluorescent signals were interactively set above the background staining. The CAIX fraction was defined as the tumor area positive for CAIX, divided by the total tumor area. The vascular parameters VD (vessel density) and RVA (relative vascular area) were defined as the number of vessels per square mm and the vascular area divided by the tumor area, respectively.

Statistics

Statistical analyses were performed on a Macintosh computer using Prism 4.0 (Hearne Scientific software, Dublin, Ireland) software package.

Correlations between CAIX fraction and tumor characteristics were tested with the Kruskal-Wallis test, or the Mann-Whitney test in case of only two subgroups. To test for differences in tumor characteristics between treatment groups the chi-square or Fisher's exact test (as appropriate) were applied. For survival analysis, patients were dichotomized by the median value of CAIX fraction and classified according to the expression pattern. Endpoints were 5-year local control, regional control, metastasis-free survival and overall survival. Univariate and multivariate Cox regression analyses were performed to evaluate the prognostic value of the different parameters. P-values <0.05 were considered significant.

Results

Paraffin embedded biopsy material could be retrieved from 303 patients. 42 biopsies were excluded because of the absence of invasive carcinoma or poor quality of the sample. In total, 261 biopsies were available for analysis. Patient and tumor characteristics are summarized in Table 1. There were no differences between the two treatment arms.

Different patterns of CAIX staining could be observed (Figure 1); a typical perinecrotic pattern, as descibed earlier by Beasley et al.[9], and a more diffuse staining pattern, unrelated to necrosis. In 187 biopsies a sufficient amount of CAIX staining was present to assess the pattern, 42 of these showing the perinecrotic pattern and 145 biopsies the diffuse pattern. The CAIX fraction defined as percentage of the total tumor area was similar for both patterns (mean 36% versus 39%, p=0.75). In 74 patients the CAIX expression was too low to recognize a pattern. Overall, the CAIX fraction varied from 0–93% (median value 22%).

Table 1 Patient and tumor characteristics.

	Paraffin (261)		Fresh frozen (79)	
	ARCON(130)	AR(131)	ARCON(40)	AR(39)
sex				
male	109 (84)	101 (77)	27 (68)	27 (69)
female	21 (16)	30 (23)	13 (32)	12 (31)
Median age (range)	62 (43-84)	61 (38-88)	61 (46-83)	60 (38-81)
T stage				
T2	42 (32)	52 (40)	5 (13)*	12 (31)
T3	72 (56)	58 (44)	30 (75)	20 (51)
T4	16 (12)	21 (16)	5 (13)	7 (18)
N stage				
N0	83 (64)	84 (64)	19 (48)	18 (46)
N+	46 (36)	47 (36)	21 (52)	21 (54)
site				
glottic	56 (43)	51 (39)	13 (33)	12 (31)
supraglottic	74 (57)	80 (61)	27 (67)	25 (64)
sub / transglottic				2 (5)
Differentiation grade				
well	9 (7)	9 (7)	2 (5)	4 (10)
moderately	77 (59)	72 (55)	23 (57)	23 (59)
poor	25 (19)	18 (14)	15 (38)	7 (18)
unknown	19 (15)	32 (24)		5 (13)
Fraction CAIX				
high	67 (52)	65 (50)	20 (50)	18 (46)
low	63 (48)	66 (50)	20 (50)	21 (54)
CAIX pattern				
Diffuse	73 (56)	72 (55)		
Perinecrotic	20 (15)	22 (17)		
No pattern	37 (28)	37 (28)		

* p = 0.07 distribution of T-stage in ARCON arm vs AR
ARCON = accelerated radiotherapy with carbogen and nicotinamide, AR = accelerated radiotherapy

Fresh frozen material was obtained from 79 patients (Table 1). The CAIX fraction varied from 0 - 26% with a median value of 0.9%. A significant correlation was observed between CAIX fraction in paraffin-embedded and fresh frozen biopsies ($r = 0.45$, $p<0.001$). Note that paraffin-embedded tissue and frozen material were processed by different staining techniques and analysis methods.

Outcome – paraffin embedded samples

No correlation was observed between CAIX fraction and tumor characteristics like T-stage, N-stage and differentiation grade.

When using the median value as a cut-off, in the group of patients treated with AR there was a trend to a worse regional control and overall survival in patients with a tumor with a low CAIX fraction compared to patients with a tumor with a high CAIX fraction (77% vs 91%, $p = 0.09$ and 48% vs 67%, $p = 0.09$ respectively, Figure 2). Patients with a low CAIX fraction treated by ARCON had a higher regional control (93% vs 77%, $p = 0.03$) and, although it did not reach statistical significance, metastasis-free survival (92% vs 79%, $p = 0.12$) than those treated by AR. In patients with a high CAIX fraction, there was no difference between treatment with ARCON and AR (regional control 95% vs 91%, $p = 0.30$ and metastasis-free survival 91% vs 87%, $p = 0.45$).

Outcome - fresh frozen material

The analysis of the frozen material confirmed the results obtained with the paraffin biopsies: improved regional control and metastasis-free survival with ARCON in patients with a low CAIX fraction (100% vs 79%, $p = 0.07$ and 100% vs 67%, $p = 0.02$, respectively). Also in this analysis no differences between the two treatments were observed in patients with a high CAIX fraction.

Staining patterns

The patients with a tumor with a perinecrotic pattern had a significantly worse local control, metastasis-free survival and overall survival (65% vs 84%, $p = 0.01$, 70% vs 96%, $p < 0.01$ and 42% vs 71%, $p < 0.01$ respectively, Figure 3) compared to the diffuse CAIX pattern. ARCON was not able to improve outcome for these patients. Consistent with the results after dichotomization by the median, the gain of ARCON over AR was predominantly seen in the group with no or very low CAIX expression and no recognizable pattern (regional control 97% vs 71%, $p = 0.01$ and metastasis-free survival 92% vs 69%, $p = 0.06$, Figure 4). Tumors with a low CAIX expression or perinecrotic pattern were more often poorly differentiated compared to the diffusely CAIX expressing tumors ($p = 0.04$), which often showed expression associated with keratinizing, well-differentiated areas. Furthermore, the percentage of tumors exhibiting a diffuse staining pattern was higher in glottic compared to supraglottic tumors, while a perinecrotic pattern was observed more often in supraglottic tumors (64% vs 49% and 21% vs 9% respectively, $p = 0.02$).

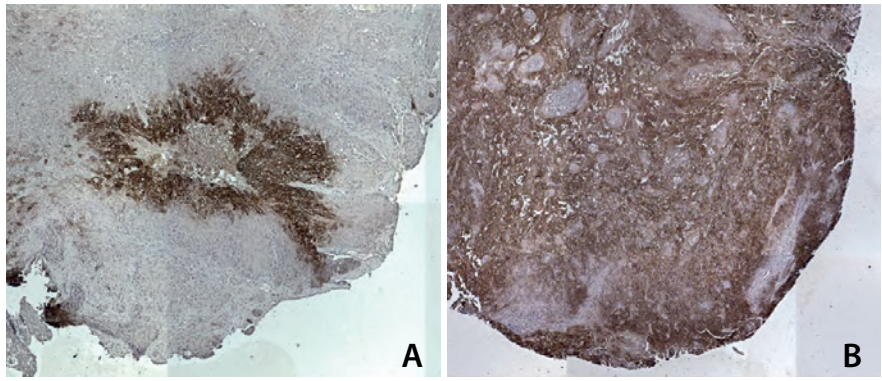

Fig. 1 Two examples of paraffin embedded biopsies, immunohistochemically stained for CAIX, showing a typical perinecrotic staining pattern (**A**) and a diffuse pattern (**B**).

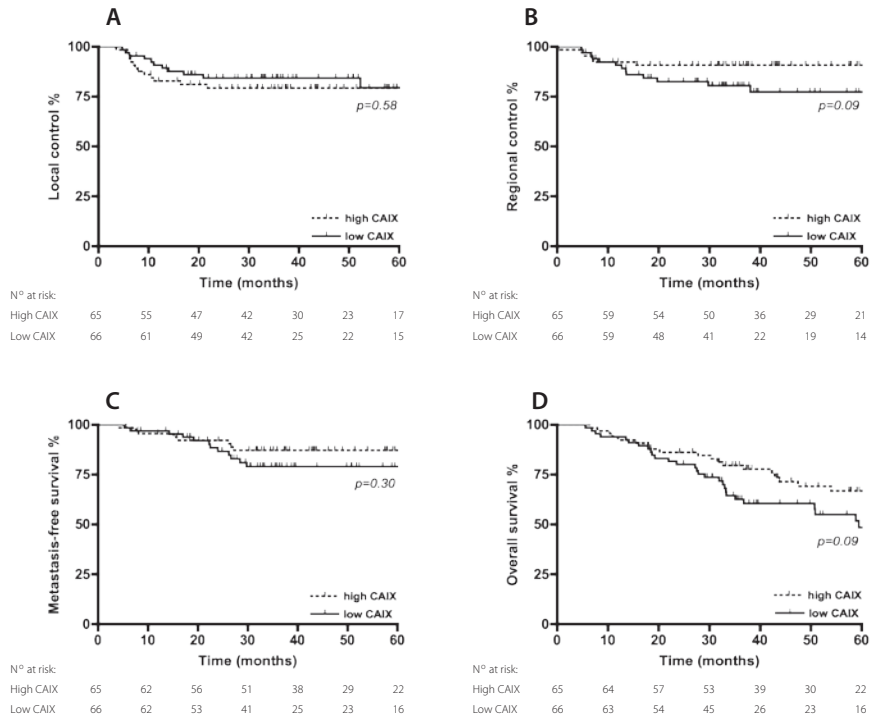

Fig. 2 Kaplan-Meier survival curves based on analysis of 261 paraffin embedded tumor biopsies showing worse regional control (**B**) and overall survival (**D**) in patients with a tumor with low expression of CAIX, treated with radiotherapy alone. No difference was observed for local control (**A**) and metastasis-free survival (**C**).

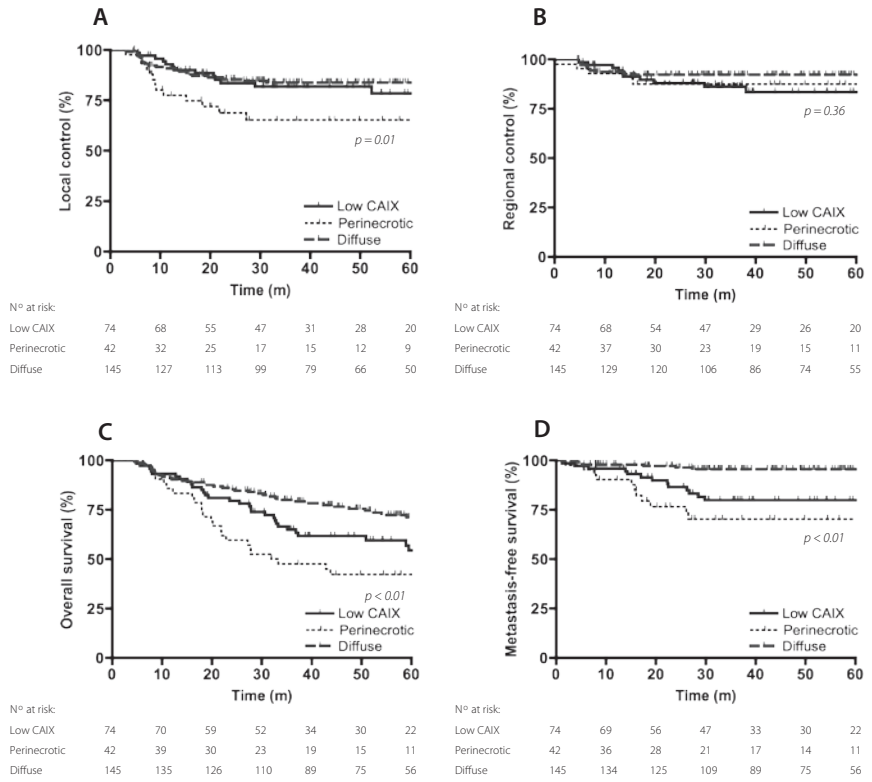

Fig. 3 Kaplan-Meier survival curves based on 261 paraffin embedded biopsies showing worse local control (**A**) metastasis-free survival (**C**) and overall survival (**D**) for patients with a tumor with a perinecrotic CAIX staining pattern compared to a diffuse pattern, irrespective of treatment. No prognostic value was seen for regional control (**B**). P-values refer to the difference between the perinecrotic expression pattern and diffuse expression pattern.

Univariate analysis including CAIX fraction, CAIX pattern, T-stage, N-stage, differentiation grade, treatment arm and differentiation grade revealed N-stage, CAIX pattern and tumor site as the most important prognostic factors for metastasis-free survival and N-stage and CAIX pattern for overall survival (Table 2). In a multivariate analysis including all significant parameters, CAIX pattern and N-stage emerged as the only significant predictors for metastasis-free survival (Table 3).

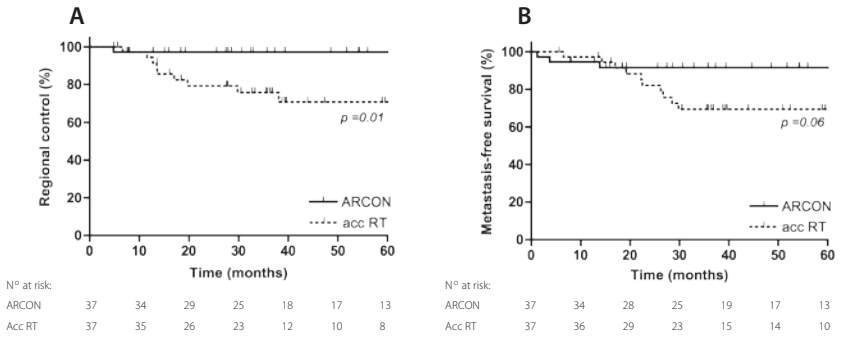

Fig. 4 Kaplan-Meier survival curves based on 74 paraffin embedded tumor biopsies with no recognizable pattern (no or very low CAIX expression) showing improved regional control (**A**) and metastasis-free survival (**B**) with ARCON.

Correlation with pimonidazole and vascular parameters

A significant, but weak correlation was observed between the CAIX fraction and the hypoxic fraction as assessed by pimonidazole staining (r = 0.36, p < 0.01, Figure 5), which has a predominant diffusion-limited staining pattern. No correlation was observed with VD (r = -0.06, p = 0.59), but a significant weak negative correlation was present with the RVA (r = -0.39, p < 0.01).

Table 2 Univariate Cox regression analysis.

		Local control			Regional control			Metastasis-free survival			Overall survival		
		HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
CAIX fraction	< median	1.0			1.0			1.0			1.0		
	> median	1.2	0.7-2.1	0.58	0.5	0.2-1.1	0.09	0.7	0.4-1.5	0.38	0.7	0.5-1.1	0.12
CAIX pattern	diffuse	1.0			1.0			1.0			1.0		
	no pattern	1.2	0.6-2.4	0.58	1.8	0.8-4.3	0.16	4.6	1.7-12.1	**0.002**	1.7	1.1-2.7	**0.03**
	perinecrotic	2.4	1.2-4.7	**0.02**	1.7	0.6-4.9	0.33	7.8	2.9-21.0	**<0.001**	2.6	1.6-4.3	**<0.001**
Diff grade	well	1.0			1.0			1.0			1.0		
	moderate	1.1	0.3-3.6	0.88	0.4	0.1-1.2	0.11	0.6	0.2-2.2	0.48	0.6	0.3-1.2	0.19
	poor	0.9	0.2-3.7	0.94	0.3	0.1-1.5	0.14	1.0	0.3-3.8	0.99	0.5	0.2-1.2	0.14
Treatment	AR	1.0			1.0			1.0			1.0		
	ARCON	1.0	0.6-1.7	0.95	0.4	0.2-0.9	**0.03**	0.5	0.3-1.1	0.10	1.0	0.7-1.5	0.92
Tumor site	glottic	1.0			1.0			1.0			1.0		
	supraglottic	0.9	0.5-1.6	0.67	1.4	0.6-3.1	0.43	2.4	1.0-5.5	**0.05**	1.2	0.8-1.8	0.42
T-stage	T2	1.0			1.0			1.0			1.0		
	T3	0.9	0.5-1.6	0.64	1.2	0.5-3.0	0.65	1.1	0.5-2.6	0.80	1.2	0.8-1.9	0.45
	T4	1.1	0.5-2.5	0.80	1.7	0.6-5.2	0.35	2.5	1.0-6.5	0.06	1.7	0.9-3.0	0.08
N-stage	N0	1.0			1.0			1.0			1.0		
	N+	1.6	0.9-2.8	0.10	3.8	1.7-8.5	**0.001**	4.0	1.9-8.5	**0.001**	1.7	1.1-2.6	**0.009**

Bold number indicates significant value

Table 3 Multivariate Cox regression analysis

		Metastasis-free survival			Overall survival		
		HR	95% CI	*p*	HR	95% CI	*p*
CAIX pattern	diffuse	1.0			1.0		
	no pattern	4.6	1.7-12.2	**0.002**	1.7	1.1-2.7	**0.03**
	perinecrotic	7.2	2.6-19.9	**<0.001**	2.5	1.5-4.1	**0.001**
Tumor site	glottic	1.0					
	supraglottic	0.8	0.3-2.2	0.69			
N-stage	N0	1.0			1.0		
	N+	4.0	1.7-9.8	**0.002**	1.6	1.1-2.4	**0.02**

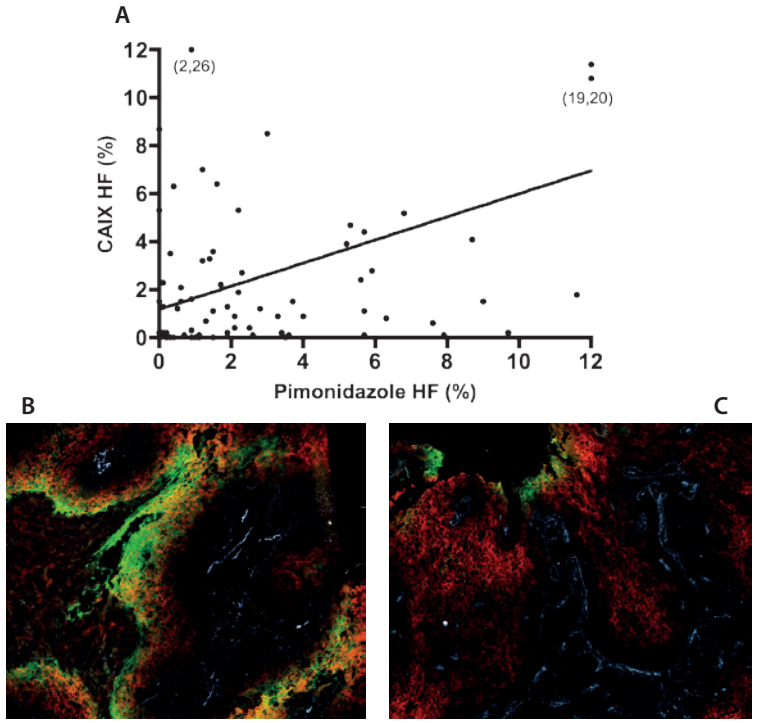

Fig. 5 (**A**) Correlation between pimonidazole hypoxic fraction and CAIX expression in 79 fresh frozen tumor biopsies (r = 0.36, p < 0.01). Linear best fit is shown. Datapoints (2,26) and (19,20) have been relocated in the graph to enhance visuality. Fluorescent microscopic image of a laryngeal carcinoma with a high (**B**) and low (**C**) degree of colocalization between pimonidazole (green) and CAIX (red).

Discussion

In this study we investigated the prognostic and predictive value of CAIX in patients with advanced laryngeal cancer treated in the ARCON randomised trial. Two datasets were used: one based on paraffin-embedded immunohistochemically stained biopsies taken for diagnostic purposes and a dataset based on additional fresh frozen biopsies obtained after injection of the hypoxia marker pimonidazole and stained with immunofluorescence. In the latter dataset multiple stainings per tissue section were performed to assess the correlation of CAIX with pimonidazole and vascular parameters. Furthermore, it provided a confirmation of the results obtained with the paraffin dataset.

Although the associations with outcome were comparable in the two datasets, the absolute values of the CAIX fraction largely differ for two reasons. The first is the difference in detection sensitivity between the two staining methods. The ABC-staining method, used on the paraffin biopsies, has a greater sensitivity, as the signal is largely enhanced by the avidin-biotinylated enzyme complexes. Secondly and more important, the method of analysis is different (parametric mapping versus binary analysis, see materials and methods section), resulting in larger fractions for the paraffin material.

Our data show predictive potential of CAIX in both datasets; regional control and metastasis-free survival was worse in patients with a low CAIX expression and this was corrected by ARCON. This may be somewhat counter-intuitive, because a higher CAIX expression is generally associated with hypoxia via HIF-1α and ARCON aims to counteract hypoxia.[5] Nevertheless, the current results are in concordance with results from a previous cohort of advanced head and neck cancer patients treated with ARCON.[10]

Although CAIX is often considered a hypoxia-associated marker, it has not shown to be very robust for quantifying clinically relevant hypoxia. The correlation with oxygen electrode measurements and exogenic hypoxic markers, like pimonidazole and EF-5, is weak as demonstrated in the current investigation as well as in other reports.[2,11-14] Studies investigating the prognostic value of CAIX have shown equivocal results, some demonstrating a negative correlation with outcome and others a positive or no correlation at all.[12,15-20]

Because of these inconsistencies we looked further into the patterns of CAIX expression as we observed striking differences in staining patterns between tumors. Three distinct types were identified: a perinecrotic pattern, a diffuse pattern and a remaining group without a recognizable pattern (very low CAIX expression). The typical perinecrotic pattern for CAIX has been described before in breast cancer and head and neck cancer as the dominant expression pattern and is generally associated with chronic or diffusion-limited hypoxia.[21,22] In the current dataset only 17% of the tumors showed this perinecrotic pattern. The diffuse CAIX expression pattern has been described earlier in VHL-defective renal cell carcinomas.[23] Patients with a tumor showing the perinecrotic pattern had a significantly worse outcome, while the diffuse pattern indicated a good

prognosis, suggesting a different biology and function of CAIX in these tumors. It should be noted that the overall amount of CAIX expression was not different between the two expression patterns. These distinct patterns have not been considered in most other studies, which could explain the contradictory results with respect to the prognostic value of CAIX. In renal cell carcinoma, which mostly show a diffuse pattern, CAIX has been found to be a positive prognostic factor as well.[24,25]

The different patterns could be the result of different mechanisms of upregulation of CAIX. The best known trigger for CAIX upregulation is through HIF-1α stabilization by hypoxia, which can be expected to play an important role in the perinecrotic expression pattern. Pathways or factors other than hypoxia can be involved in HIF-1α stabilization and CAIX upregulation in normoxic circumstances. These include the PI3-K pathway [26], acidosis [27,28] and genetic alterations like in renal cell carcinomas. VHL or p53 mutations can inhibit the degradation of HIF-1α [29,30], which, upon stabilization, stimulates expression of CAIX. These explanations for the more diffuse, hypoxia-independent, staining pattern do not necessarily imply a worse prognosis with higher CAIX expression.

Furthermore, this study comprises only a distinct subgroup of all head and neck cancers i.e. laryngeal carcinoma. Etiological factors differ between the specific head and neck subsites.[31,32] Previous investigations showed that laryngeal carcinomas harbour a restricted spectrum of p53 mutations [33] and are often more well differentiated than other head and neck subsites. Such differences can have major impact on the expression of tumor markers and the response to therapy.

The correlation with differentiation grade, with well-differentiated keratinizing tumors exhibiting more often a diffuse pattern and high grade tumors a low CAIX expression or perinecrotic pattern leads to another hypothesis. Well-differentiated tumors could be expressing CAIX as a physiological reaction to an altered microenvironment (changes in pH due to rapid tumor growth and altered metabolism for example). The loss of this reactive diffuse CAIX expression in tumors with low or perinecrotic CAIX expression could be a sign of dedifferentiation and more aggressive behavior including metastasis formation. However, only in the low CAIX tumors this can be counteracted by ARCON. This indicates that perinecrotic CAIX signifies an aggressive tumor phenotype and that this aggressiveness may not necessarily be related to hypoxia. Furthermore, it can be concluded that absence of CAIX does not implicate absence of hypoxia. This is supported by only a weak correlation between CAIX expression and pimonidazole binding and the ability of ARCON to improve outcome in tumors with low CAIX expression.

Another study employing hypoxic (radio)sensitisation using the nitroimidazole compound nimorazole showed no predictive value of CAIX.[34] However, different subsites of head and neck cancer were included in that study, the pattern of CAIX expression was not assessed and the mechanism of action of nimorazole differs from that of ARCON which may all be explanations for the discrepancy with the current study.

Interestingly, studies investigating HIF-1α have found the same prognostic value of the pattern of marker expression. A perinecrotic HIF-1α expression was a negative prognostic factor compared to a diffuse pattern in studies in breast cancer and endometrial carcinoma[21,35] and regarded as a sign of tumor agressiveness.

Conclusion

ARCON improves regional control and metastasis-free survival in patients with a larynx carcinoma with low expression of CAIX. A diffuse CAIX staining pattern is associated with tumor differentiation and better outcome results. A perinecrotic CAIX pattern is indicative for an agressive phenotype with high risk of metastasis formation, that cannot be counteracted with ARCON treatment.

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6

Prognostic value of the proliferation marker Ki-67 in larynx carcinoma: results of the ARCON phase III randomized trial

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Abstract

Purpose
The prognostic and predictive value of the proliferation marker Ki-67 was investigated in a phase III trial in patients with T2-4 laryngeal carcinoma comparing ARCON (accelerated radiotherapy with carbogen breathing and nicotinamide) to accelerated radiotherapy alone (AR).

Methods
Tumor biopsies from 255 patients were immunohistochemically stained for Ki-67 and the hypoxia-related marker CAIX. Labeling index of Ki-67 (Ki-67-LI) and the colocalization with CAIX was related to tumor control and patient survival.

Results
On average, node positive patients had a higher Ki-67-LI compared to node negative patients (median 14% vs 8% $p < 0.01$). In the cohort treated with AR alone high Ki-67-LI (>10%) was associated with increased regional and distant metastases formation. This was not observed in the patients treated with ARCON. Regional- and distant metastases-free survival were 79% vs 96%, $p < 0.01$ and 71% vs 88% $p = 0.05$, for AR and ARCON, respectively. Local control and disease-specific survival were not significantly different between the treatment arms (78% vs 80%, $p = 0.91$ and 70% vs 76%, $p = 0.53$). Patients with low Ki-67 expression had an excellent outcome with accelerated radiotherapy and this could not be further improved with ARCON. Colocalization of Ki-67 with CAIX provided no additional prognostic information.

Conclusion
Patients with larynx carcinomas with high proliferative activity are at increased risk of regional and distant metastases formation. This risk can be reduced by treatment with ARCON.

Background

Early stages of laryngeal cancer can, in most cases, be cured with laser surgery or radiotherapy alone. For the more advanced stages, radiotherapy is the treatment of choice either or not combined with chemotherapy, but targeted therapies and hypoxia-modifying treatments are applied as well. Given that only a minority of the patients profits from these modifications, research efforts should focus on biology-based selection of patients for the most effective treatment and least burden.

A well-known adverse prognostic factor in all types of cancer is proliferation. A meta-analysis showed a correlation of high proliferation index with low survival in head and neck cancer, albeit individual studies show equivocal results.[1] A widely used immuno-histochemical proliferation marker is Ki-67. This endogenous marker is expressed in all phases of the cell cycle, with exception of the G0 phase and is thought to represent the growth fraction of the tumor.

Another extensively studied adverse prognostic factor is hypoxia. Hypoxic tumors are more resistant to therapy and have a worse outcome compared to well-oxygenated tumors.[2,3] In theory, hypoxic cells that have retained their proliferative capacity could be the cause of persistent or recurrent disease. A previous study showed the presence of such a subpopulation of tumor cells and its correlation with disease-free survival.[4]

To specifically target both proliferation and hypoxic radioresistance, ARCON (accelerated radiotherapy with carbogen breathing and nicotinamide) treatment was introduced, which combines accelerated radiotherapy with carbogen breathing and nicotinamide. A randomized phase III trial in the Netherlands in 345 patients with advanced carcinoma of the larynx showed increased regional control with ARCON.[5] In the current study, the biopsy material from these patients has been used to assess the prognostic value of Ki-67 either as a single marker or combined with the hypoxia-associated marker carbonic anhydrase IX (CAIX) and the response to ARCON therapy.

Materials and methods

Patients
In the ARCON phase III trial 345 patients with advanced laryngeal cancer were randomized between accelerated radiotherapy (AR) and ARCON from 2001 to 2008. Inclusion criteria were: histological confirmation of squamous cell carcinoma of the larynx, stage T2b-T4, all N-stages, no distant metastasis, WHO performance status 0-1, age > 18 years, and written informed consent. Radiotherapy in the experimental arm was combined with carbogen (98% O_2 and 2% CO_2) breathing and nicotinamide (60 mg/kg orally) administration 1-1.5 h before treatment. Biopsy material obtained for routine purposes of 303 of these patients was retrieved from 37 pathology departments in the Netherlands. The biopsies had been

fixed in formaldehyde and paraffin-embedded. All samples were cut in sections of 5 µm. One section was stained for haematoxylin and eosin as a guide to delineate the tumor area.

Immunohistochemistry

Paraffin sections were deparaffinized in Histosafe (clearing agent, Adamas, the Netherlands), rehydrated through a graded ethanol series and boiled for 30 minutes in antigen retrieval solution. After cooling for 25 minutes and rinsing in PBS, endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Then, sections were incubated with 5% normal donkey serum in PAD (primary antibody diluent, Abcam, UK) to block non-specific binding.

Sections were incubated at 4 °C overnight with rabbit anti-human Ki-67 (Abcam, UK) diluted 1:50 in PAD. Subsequently, the biotinylated secondary antibody was applied; biotin-labelled-F(ab')$_2$-donkey anti-rabbit IgG (Jackson Immuno Research), followed by ABC-reagent (Vector Elite kit, Vector Laboratories) for 30 minutes.

For the detection of CAIX, consecutive sections were incubated at 4 °C overnight with mouse anti-CAIX (E. Oosterwijk, department of Urology, Radboud University Nijmegen Medical Centre) diluted 1:25 in PAD, followed by labeling with biotin-labelled-F(ab')$_2$-donkey anti-mouse IgG (Jackson Immuno Research) as a secondary antibody and ABC-reagent (Vector Elite kit, Vector Laboratories) for 30 minutes.

Peroxidase activity was detected with diaminobenzidine (DAB). Finally, sections were counterstained with haematoxylin for 30 seconds and mounted with KP mounting medium (Klinipath, Duiven, the Netherlands). For negative controls, PAD was added without the primary antibody.

Image processing

Whole tumor sections of the paraffin-embedded biopsies were scanned and analyzed by parametric mapping as described before.[6] With this method, besides analysis of single markers, colocalization of multiple markers in different subcellular compartments can be assessed. In short, a mosaic image of a whole tumor section is subdivided in squares of 36 x 36 µm. For each square the labeling index of Ki-67 was determined and set as a new value in the resulting parameter image map. This value was compared with the value of the corresponding square in the CAIX parameter image map, that was labeled positive or negative for CAIX.

The labeling index of Ki-67 (Ki-67-LI) was calculated as well as the area positive for CAIX relative to the total tumor area (fraction CAIX). The colocalization of Ki-67 with CAIX was assessed using two parameters: $FCAIX_{[Ki-67]}$ (Fraction of area positive for CAIX and for Ki-67 relative to total area positive for CAIX) and $FKi\text{-}67_{[CAIX]}$ (Fraction of area positive for CAIX and for Ki-67 relative tot total area positive for Ki-67).

The pattern of CAIX staining was visually scored, distinguishing a perinecrotic pattern, a diffuse pattern or no recognizable pattern due to very low CAIX expression.

Statistics

Statistical analyses were performed on a Macintosh computer using Prism 4.0 (Hearne Scientific software, Dublin, Ireland) software package. Differences between tumor characteristics and markers were tested with the Mann-Whitney test or in case of multiple groups the Kruskal-Wallis test. Correlations between markers were calculated using the Spearman rank correlation test. For survival analysis marker parameters were dichotomized based on the median value. Local control (LC), regional control (RC), metastasis-free survival (MFS) and disease-specific survival (DSS) were estimated using the Kaplan-Meier method and compared with the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. P-values < 0.05 were considered significant.

Results

From the 303 paraffin biopsies, 255 biopsies were suitable for analysis. 48 biopsies were excluded for various reasons: no or very scarce tumor tissue in the biopsy, tissue section damaged during staining procedure or poor staining quality. Tabel 1 shows the patient and tumor characteristics. The median Ki-67-LI was 10% with a range of 0 - 44%. Examples of the staining are shown in Figure 1.

Colocalization of Ki-67 with CAIX varied from 0 – 100% ($FCAIX_{[Ki-67]}$) and 0 - 71% ($FKi\text{-}67_{[CAIX]}$) and correlated very strong with the single marker expression ($FCAIX_{[Ki-67]}$ vs Ki-67-LI $r = 0.82$, $p < 0.01$ and $FKi\text{-}67_{[CAIX]}$ vs fraction CAIX $r = 0.87$, $p < 0.01$).

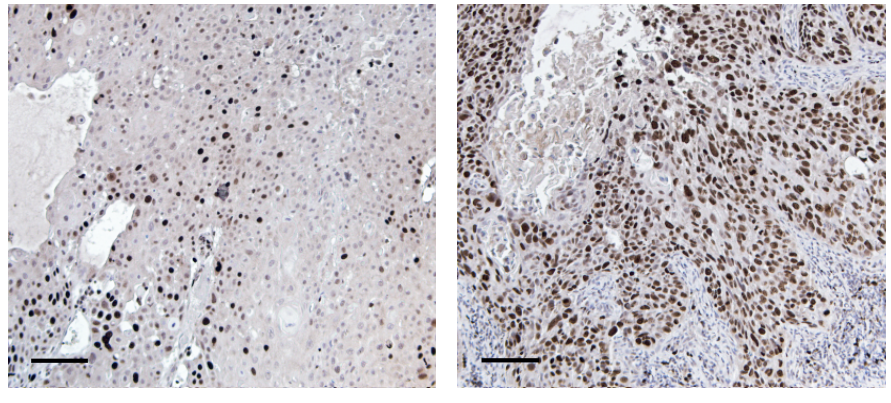

Fig. 1 Examples of immunohistochemical staining for Ki-67 expression with a haematoxylin counterstain. Tumor with a low Ki-67 labeling index (8%; **A**) and a high Ki-67 labeling index (17%; **B**). Scalebars: 100 mm.

Table 1 Patient and tumor characteristics.

	ARCON N=127 (%)	AR N=128 (%)
sex		
male	107 (84)	97 (76)
female	20 (16)	31 (24)
Median age (range)	61 (42-84)	60 (38-88)
T stage		
T2	41 (32)	50 (39)
T3	70 (55)	57 (45)
T4	16 (13)	21 (16)
N stage		
N0	82 (65)	82 (64)
N+	44 (34)	46 (36)
Unknown	1 (1)	
site		
glottic	55 (43)	49 (38)
supraglottic	72 (57)	79 (62)
Differentiation grade		
well	9 (7)	9 (7)
moderately	75 (59)	71 (55)
poor	24 (19)	18 (14)
unknown	19 (15)	30 (23)
Ki-67-LI		
high (>10%)	67 (53)	59 (46)
low (≤10%)	60 (47)	69 (54)

Abbreviations: AR, accelerated radiotherapy; ARCON, accelerated radiotherapy combined with carbogen and nicotinamide; Ki-67-LI, labeling index of Ki-67

Correlation with clinicopathological characteristics

On average, node positive patients had a significantly higher Ki-67-LI than node negative patients (median 14% vs 8% $p < 0.01$, Figure 2). Higher T-stage was associated with a slightly higher Ki-67-LI (7% for T2 and 11% for T3/4, $p = 0.09$).

Ki-67-LI was not correlated with fraction CAIX ($r = 0.10$, $p = 0.14$). Ki-67-LI of tumors with a perinecrotic CAIX staining pattern was higher compared to tumors with a diffuse CAIX staining pattern (median 14% vs 9%, $p = 0.02$).

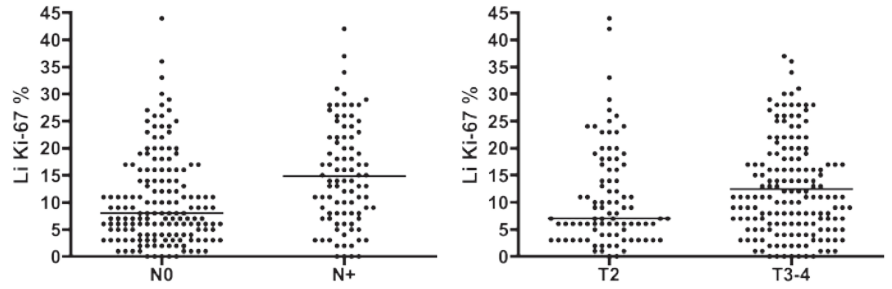

Fig. 2 Labeling index of Ki-67 is higher in patients with positive lymph nodes (**A**) or higher T-stage (**B**) at diagnosis.

Associations with treatment outcome

With accelerated radiotherapy alone, patients with a high Ki-67-LI (>10%) had a worse RC (79% vs 91%, $p = 0.08$), MFS (71% vs 96%, $p < 0.01$) and DSS (70% vs 89%, $p < 0.01$, Fig. 3) compared to those with a low Ki-67-LI. In patients with high Ki-67-LI RC was significantly better with ARCON compared to AR (96% vs 79%, $p < 0.01$, Fig. 4) and an improvement in MFS was observed as well (88% vs 71% $p = 0.05$). ARCON had no effect on LC and DSS (80% vs 78%, $p = 0.91$ and 76% vs 70%, $p = 0.53$ for ARCON vs AR, respectively). Patients with low Ki-67 expression had an excellent outcome irrespective of the treatment arm (Figure 3 and 4). In the subgroup of patients with no nodal metastasis at diagnosis (N0), predictive value of Ki-67-LI was maintained for regional control (ARCON 100% vs AR 85%, $p = 0.04$).

Table 2 shows the results of the univariate analysis separate for both treatment arms. In a multivariate analysis including Ki-67-LI and N-stage, Ki-67-LI lost its trend to significance with respect to regional control. However, the effect on MFS remained significant for N-stage as well as for Ki-67-LI. Both parameters showed a trend to significance for DSS ($p = 0.06$ and $p = 0.05$ respectively) in multivariate analysis.

The results obtained with Ki-67 / CAIX colocalization parameters were similar to the single marker analysis and provided no additional prognostic information.

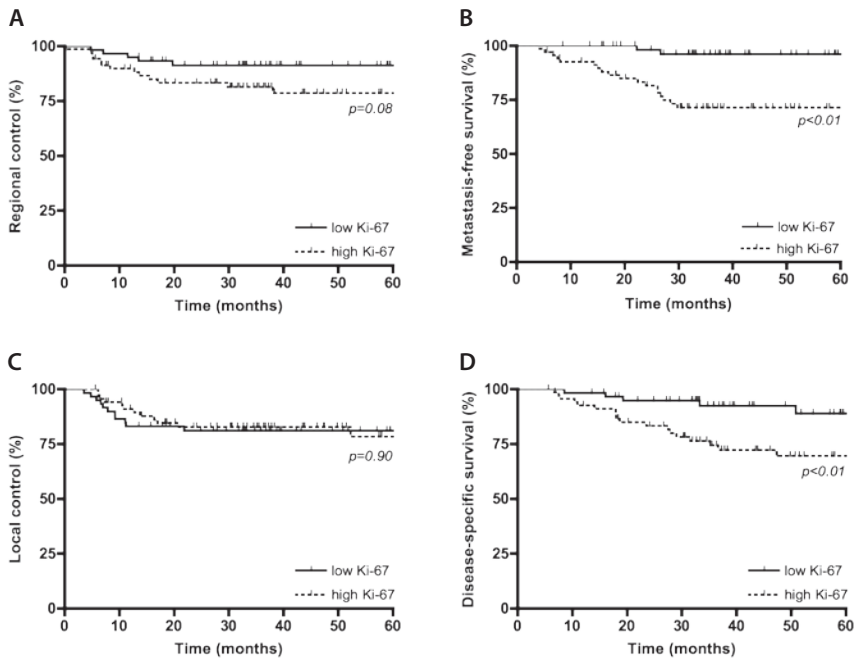

Fig. 3 Kaplan Meier survival curves for regional control (**A**), metastasis-free survival (**B**), local control (**C**) and disease-specific survival (**D**) in patients treated with accelerated radiotherapy dichotomized by the median Ki-67 labeling index.

Table 2 Univariate cox regression analysis.

Parameter	Local control		Regional control		Metastasis-free survival		Disease-specific survival	
	AR	ARCON	AR	ARCON	AR	ARCON	AR	ARCON
N-stage	0.71*	0.07	**0.02**	0.05	**0.002**	0.08	**0.01**	**0.003**
Ki-67	0.90	0.63	0.09	0.36	**0.004**	0.36	**0.01**	0.13
T-stage	0.63	0.52	0.63	0.40	0.05	0.94	**0.03**	0.46
Grade	0.67	0.18	0.70	0.23	0.32	0.40	0.45	0.41
Site#	0.14	0.11	0.66	0.08	0.44	0.76	0.68	**0.04**

Abbreviations: AR, accelerated radiotherapy; ARCON, accelerated radiotherapy combined with carbogen and nicotinamide

* p-values are given, bold number indicates significant value # glottic or supraglottic

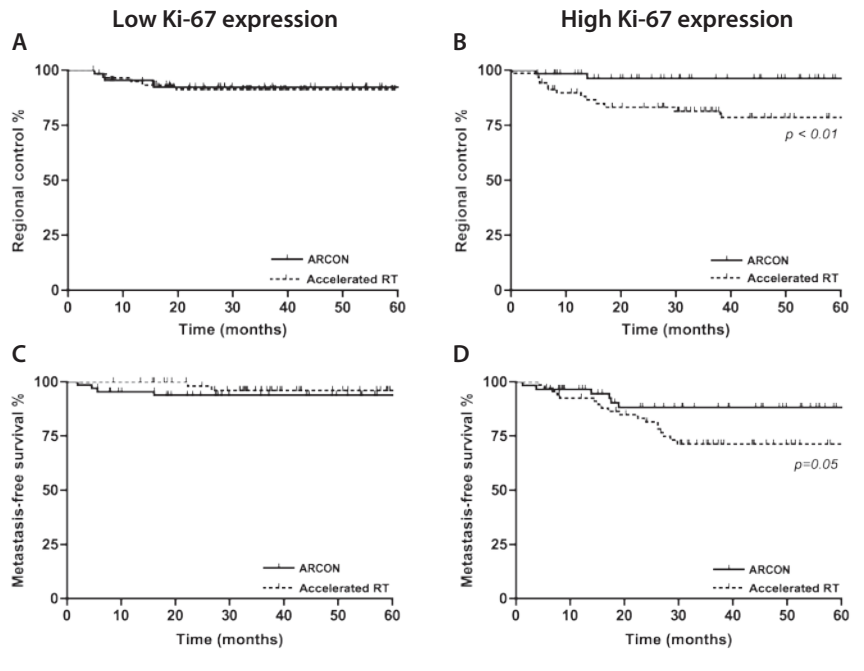

Fig. 4 Kaplan Meier survival curves show increased regional control and metastasis-free survival with ARCON in patients with high Ki-67 expression (**B** and **D**). No difference was observed in patients with tumors with low Ki-67 expression (**A** and **C**).

Discussion

In this study we showed that larynx cancer patients treated with accelerated radiotherapy alone and a high Ki-67-LI have an increased risk of regional and distant metastases formation compared to patients with a low Ki-67-LI. In addition, we showed that ARCON reduces the incidence of nodal and distant metastatic failures in patients with tumors with high proliferative activity.

Ki-67 expression has been correlated to advanced tumor stage at diagnosis and poor histological grading, [7-9] emphasizing the relationship between tumor agressiveness and proliferation. Furthermore, Ki-67 has been correlated to clinical outcome in numerous studies. The prognostic significance of Ki-67 expression is well established in breast cancer and to a lesser extent also in lung cancer and soft tissue tumors.[10] Several studies assessed the prognostic value of Ki-67 in laryngeal cancer specifically, but mostly in surgically treated patients.[7,11,12] Some of those have shown a correlation of Ki-67-LI with recurrence

risk, albeit the exact site of recurrence was not always mentioned.[1] One study showed prognostic value of high Ki-67 for the occurrence of distant metastasis, consistent with findings of the current investigation.[13] Results from two randomized trials comparing accelerated radiotherapy versus conventional radiotherapy in head and neck cancer indicate that tumors with low expression of proliferation markers profit most from accelerated radiotherapy regimes with respect to local control.[8,14,15] So, low Ki-67 identifies patients that could benefit from accelerated radiotherapy to improve local tumor control, whereas high Ki-67 is indicative for a metastatic phenotype.

In the current study, all patients received accelerated radiotherapy, and no prognostic value was seen for Ki-67 expression on local control, as can be expected based on the previously discussed literature data. However, high Ki-67 expression was correlated with worse RC and MFS in patients treated with accelerated radiotherapy alone. So, accelerated radiotherapy does not correct for the negative prognostic value of Ki-67 on these endpoints, while the addition of hypoxic modification does. How can this be explained?

There is a mutual relationship between proliferation and hypoxia: highly proliferating tumors will rapidly outgrow their vascular supply thus creating hypoxic areas. Furthermore, hypoxic conditions can drive a cell towards a more agressive phenotype with invasive and migratory potential and more regional and distant metastasis.[16] Malignant tumors display gradients of hypoxia. Tumor cells at larger distance from the blood vessels under severe hypoxia show no or very low proliferative activity.[4,17,18] Intermediate levels of hypoxia can harbor an aggressive population of tumor cells, that have a larger capability of proliferation and formation of metastasis (regional or distant). This intermediate hypoxic cell population may be more relevant for the effect of ARCON than the severely hypoxic cells. This is why we analyzed colocalization between Ki-67 and CAIX as a marker for intermediate hypoxia. Although we did observe an association between colocalization parameters and metastases formation and effect of ARCON, the predictive power was not better than that of Ki-67 as a single marker. This could be due to the fact that CAIX expression is not only related to hypoxia. In the current material we observed a diffuse (hypoxia-unrelated) expression pattern in 56% of the laryngeal tumors (data not shown).

Most studies report locoregional control as an outcome measure for head and neck cancer. However, differentiation between local and regional control might be more appropriate as local failure is likely caused by different mechanisms than regional and distant failures. For a tumor to spread through lymph and blood vessels, cells must have migratory capacity by transformations like epithelial mesenchymal transition. Hypoxia can drive tumor cells towards this migratory phenotype and at the same time protect these cells from radiation-induced injury. Hence, a beneficial effect of hypoxia modifying treatment can be expected through sensitization of this critical cell population. A local recurrence can be caused by radioresistent cells with a large capacity for repair and repopulation, in which hypoxia might play a minor role. Indeed, it has been suggested that well differentiated tumors have this ability more than poorly differentiated tumors.[19,20]

High radiation dose and accelerated radiotherapy schedules can overcome this resistance, while hypoxic modification can be beneficial for patients who have tumors with a more metastasizing phenotype.

Conclusion

High Ki-67 expression is a poor prognostic factor for regional control and metastasis-free survival in patients with advanced laryngeal cancer treated with accelerated radiotherapy. The addition of hypoxic modification with ARCON reduces regional and distant metastatic failures achieving excellent cure rates comparable to that of patients with low proliferative tumors.

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7

General Discussion

Discussion

This thesis investigated the addition of carbogen breathing and nicotinamide to accelerated radiotherapy (ARCON) in patients with advanced laryngeal cancer and the possibilities to select the patients that will benefit most from this approach.

Hypoxic modification and patient selection

The adverse prognostic impact of hypoxia in head and neck cancer prompted several approaches to counteract this resistance mechanism. As multiple methods are available to measure hypoxia in tumors, these hypoxia-modifying treatments are often combined with the assessment of the hypoxic state of the tumor to select the patients who will benefit from the hypoxia-modifying treatment.

Chapter 2 describes the results of a randomized trial comparing accelerated radiotherapy alone with ARCON. There was no significant difference in the primary endpoint, local control rate, while the 2- and 5-year regional control rates were significantly better with ARCON. Important to note on this point is the fact that in the patients treated with ARCON the total dose on the larynx was 64 Gy instead of 68 Gy to reduce the risk of laryngeal necrosis. In the patients with hypoxic tumors, based on pimonidazole staining, ARCON treatment resulted in a regional control rate of 100% compared to 55% with accelerated radiotherapy alone, whereas no difference was seen in the patient group with non-hypoxic tumors, emphasizing the importance of patient selection.

Several other strategies of patient selection for hypoxia-modifying treatments have been attempted over the last years.

In a side study of the DAHANCA 5 randomized trial, studying the addition of nimorazole to radiotherapy in head and neck cancer, the investigators showed that the plasma level of osteopontin, as an indirect measure for tumour hypoxia, can be indicative for the respons to hypoxic sensitization.[1] Only patients with high plasma levels of osteopontin profited from the addition of nimorazole. The HPV-status of the patient is a possible selection tool as well, with a significant gain of oxygen modification in HPV-negative patients, but not in HPV-positive patients.[2] A stronger prediction model making use of a hypoxia gene expression classifier has been published recently. Only HPV-negative patients with hypoxic tumors based on the 15-gene hypoxia classifier profited from the addition of nimorazole, resulting in a significantly higher locoregional control and disease-specific survival.[3,4] Attempts have been made to predict the response to tirapazamine with osteopontin, hepatocyte growth factor and interleukin-8 with negative results.[5,6] Rischin et al. demonstrated the feasibility of selecting patients for tirapazamine treatment based on (18F)-fluoromisonidazole-PET scanning,[7] but no randomized trial has been conducted with this concept. The phase III trial with tirapazamine in an unselected patient population showed no benefit of hypoxic modification, emphasizing the importance of patient selection in large randomized trials.

To conclude, hypoxia-modifying therapies are effective in head and neck cancer. A meta-analysis of randomized trials applying oxygen-enhanced radiotherapy showed increased locoregional control, disease-specific survival and overall survival.[8] However, it is indispensable to select the patients that will profit most from hypoxia-modifying treatments using direct or surrogate markers of tumor hypoxia.

Radiotherapy combined with chemotherapy and targeted therapies

Nowadays, the standard treament for advanced head and neck cancer, including laryngeal cancer, is chemoradiation. It is important to note that no biological markers are available tot predict the respons to chemoradiation.[9] The selection of patients is based on clinical characteristics like tumor volume, nodal stage and performance status. HPV status is currently under investigation as a possible selection method for radiotherapy or combined treatment.

A plethora of targeted therapies has been developed and many are tested in clinical studies. In head and neck cancer the best known is cetuximab, an antibody directed against the epidermal growth factor receptor. In a randomized phase III trial in patients with advanced head and neck cancer 5-year-survival was higher with combined treatment (46% vs 36% with radiotherapy alone).[10] Unfortunately, increased skin toxicity is reported with cetuximab[11] and no validated selection tool is available at the moment.

Many targeted therapies fail in the majority of patients due to tumor heterogeneity and evasion mechanisms. The strength of ARCON is that it specifically tackles hypoxia, a general resistance mechanism.

Predictive markers for ARCON

In this thesis several markers have been evaluated on their relation with hypoxia and their applicability as predictive markers.

Pimonidazole

Although the hypoxic fraction as assessed by the exogenous marker pimonidazole staining as described in Chapter 2 can predict the response to ARCON, it has some practical drawbacks for implementation in clinical practice. Pimonidazole has to be administered intravenously before biopsy taking, at a time that the diagnosis is not confirmed yet. Furthermore, with the quantitative method we described additional fresh frozen biopsies are needed. The method of analysis is highly suited for a large series of biopsies, but less suitable for assessment of the hypoxic state of an individual biopsy, due tot day-to-day variation in staining and thresholding. Recently, an oral formulation of pimonidazole has become available, that can circumvent some of the drawbacks. Pimonidazole staining on paraffin biopsies should be validated and this could be implemented in daily practice.

Even though the presence of a tumor cell population staining positive for pimonidazole points at tumor cell adaptation enabling survival under hypoxic conditions, it is important to realize that pimonidazole is a chemical assessment of hypoxia, and contains no information on the biological response of the tumor cell to the hypoxic conditions.

Endogenous hypoxia-related markers

Above mentioned drawbacks do not apply to endogenous markers; paraffin embedded biopsies taken for routine diagnostic purposes can be used to stain several markers. Endogenous hypoxia-related markers, alone or in combination, could be candidates to predict the response to ARCON treatment and can further clarify the cellular response to hypoxia. In Chapter 3, a method for analysis of colocalization of markers in different subcellular compartments is described as this can be more informative on the function of a protein than analysis of a single marker. The combination of CAIX and Ki-67 was investigated, as a previous study showed prognostic value of a subpopulation of proliferating cells under hypoxic conditions.[12] The method can be applied with numerous other markers. An additional finding was the good correlation of the CAIX fraction and Ki-67 labeling index between multiple biopsies of a same tumor. Often, it is questioned whether the analysis of a single tissue section is representative due to the intratumoral variation. The results from this chapter indicate that a single section provides a good approximation, at least for the markers investigated.

CAIX

Against expectations, the combination of CAIX and Ki-67 showed no additional value as a prognostic or predictive factor compared to each of the markers alone (Chapter 6). This could be due to the fact that CAIX is not only a hypoxia-related marker as hypothesized in Chapter 5. In this chapter the relevance of the pattern of CAIX is described. In some tumors CAIX shows a typical perinecrotic pattern, that seems hypoxia-related and in other tumors a more diffuse pattern is observed, often in well-differentiated keratinizing areas, that seems not related to hypoxia. The perinecrotic pattern is the one most described in the literature[13] and indicates worse patient outcome with higher CAIX expression. The diffuse pattern has been described before in renal cell carcinoma, with a reversed prognostic value of CAIX.[14] Considered in that light, the reversed prognostic and predictive value of CAIX in the ARCON randomised trial (Chapter 5, worse regional control and metastasis-free survival in patients with a low CAIX expression, correction by ARCON) is less surprising as 145 of the 187 CAIX-positive tumors exhibited a diffuse CAIX staining pattern. The different patterns could be the result of different mechanisms of upregulation of CAIX. The best known trigger for CAIX upregulation is through HIF-1α stabilization by hypoxia, which can be expected to play an important role in the perinecrotic expression pattern. Pathways or factors other than hypoxia can be involved in HIF-1α stabilization and CAIX upregulation in normoxic circumstances, like PI3-K pathway activation,[15] acidosis[16,17] and

genetic alterations, e.g. VHL or p53 mutations. This explains the often weak correlation with clinically relevant hypoxia.[18-20] This diffuse pattern could be more for laryngeal carcinoma, as it has not been described in studies including other head and neck sites.

So, CAIX can predict the respons to ARCON therapy in advanced laryngeal cancer, although in a different way than expected beforehand. Furthermore the CAIX staining pattern seems indicative for the tumor phenotype and predicts outcome.

Proliferation and the response to ARCON

Accelerated radiotherapy improves local control compared to conventionally fractionated radiotherapy.[21,22] An important cause of treatment failure is accelerated repopulation occuring during the course of radiation treatment.[23] To overcome this resistance, treatment time is shortened from seven to five or six weeks.

Although repopulation is related to proliferation, the ability of the tumor to repopulate effectively will not solely depend on the proliferation status of the tumor before treatment, but also on the ability of the tumor to react to radiotherapy-induced cell kill. It has been suggested that the ability to accelerate repopulation is a characteristic of well to moderately differentiated tumors and may be lost by dedifferentiation.[24]

High proliferation status has been often described as associated with advanced tumor status and is an adverse prognostic factor in many studies.[25] Ki-67, an endogenous proliferation marker, is widely used, mostly in surgical series. but several studies have investigated the relation between proliferation status of the tumor and response to radiation as well,[26,27] and conclude that tumors with low proliferative activity benefit most from accelerated treatment. In the standard arm of the ARCON trial (accelerated radiotherapy alone) patients with low Ki-67 expression indeed did have an excellent local and regional control (Chapter 6). However, in patients with high Ki-67 expression an increased rate of regional and distant metastasis was observed with AR, while the addition of hypoxic modification corrects for this negative prognostic factor. The explanation for this phenomenon could be found in the mutual relationship between hypoxia and proliferation: highly proliferating tumors will rapidly outgrow their vascular supply thus creating hypoxic areas. Furthermore, hypoxic conditions can drive a cell towards a more agressive phenotype with invasive and migratory potential and more regional and distant metastasis.[28] Malignant tumors display a gradient of hypoxia. Tumor cells at larger distance from the blood vessels under severe hypoxia show no or very low proliferative activity.[12,29,30] Intermediate levels of hypoxia can harbor an aggressive population of tumor cells, that have a larger capability of proliferation and formation of metastasis (regional or distant). This intermediate hypoxic cell population may be more relevant for the effect of ARCON than the severely hypoxic cells.

Site of recurrence

Most studies report locoregional control as an outcome measure for head and neck cancer. However, differentiation between local and regional control might be more appropriate as local failure is likely caused by different mechanisms than regional and distant failures. A local recurrence can be caused by radioresistent cells with a large capacity for repair and repopulation. It has been suggested that well differentiated tumors have this ability more than poorly differentiated tumors.[22,24] High radiation dose and accelerated radiotherapy schedules can overcome this resistance. In hypoxic tumors radioresistent cells in hypoxic areas can play an important role in local recurrence as well and in patients with these tumors hypoxia modifying treatment can be beneficial.

For a tumor to spread through lymph and blood vessels, cells must have migratory capacity by transformations like epithelial mesenchymal transition.[31] Hypoxia can drive tumor cells towards this migratory phenotype[32] and at the same time protect these cells from radiation-induced injury, leading to regional and distant failure. Hence, a beneficial effect of hypoxia modifying treatment on regional and distant control can be expected through sensitization of this critical cell population in patients who have hypoxic tumors with a more metastasizing phenotype.

Other head and neck tumor sites

This thesis comprises only a distinct subgroup of all head and neck cancers i.e. laryngeal carcinoma. Etiological factors differ between the specific head and neck subsites.[33,34] Previous investigations showed that laryngeal carcinomas harbour a restricted spectrum of p53 mutations[35] and are often better differentiated than other head and neck subsites. Such differences can have major impact on the expression of tumor markers and the response to therapy. Most studies include patients with different subsites of head and neck cancer. However, the discovery of HPV as an etiological factor in oropharyngeal cancer emphasizes the differences in tumor biology and the heterogeneity of this type of cancer.

Future considerations

As more treatment options become available for patients with laryngeal cancer and more knowledge is gained about the variation in tumor biology, selection of patients for the most beneficial treatment is indispensable.

It seems that the typical well-differentiated tumor with low Ki-67 expression and diffuse CAIX staining already profits from accelerated radiotherapy with respect to local control and has a low chance of metastasizing. The typical poorly differentiated tumor with high ki-67 expression and low CAIX staining profits from hypoxic modification with ARCON with respect to RC and MFS. Perinecrotic CAIX staining is a poor prognostic factor, especially for the development of distant metastasis, perhaps these patients could profit from chemoradiation or adjuvant chemotherapy.

The last question is, if we really need a hypoxic marker if more agressive tumors in general (although often characterized by hypoxia) profit from ARCON. A simple predictive assay based on TNM stage, differentiation, proliferation and hemoglobin levels (data not published yet) could be an excellent tool to select patients for this hypoxia-modifying treatment with low toxicity. However, until now, pimonidazole is the strongest predictor for the response to ARCON.

Although this thesis investigates ARCON in laryngeal cancer specifically, this treatment has the potential to improve the outcome of all tumors with an hypoxic phenotype, irrespective of tumor site, e.g. other head and neck sites, gynaecological and bladder tumors. Hypoxia is an adverse prognostic factor in all head and neck cancer subsites and hypoxic modification with nimorazole has shown to improve outcome of tumors at different subsites. Furthermore, the phase II ARCON trial showed an excellent outcome in advanced oropharyngeal carcinomas besides carcinomas of the larynx.

In bladder cancer addition of carbogen breathing and nicotinamide to radiotherapy has already shown improved local control and overall survival,[36] making it an organ-preserving alternative to radical cystectomy. In gynaecological cancer most cervical tumors are squamous cell carcinomas, the most prevalent histology in head and neck cancer as well. Furthermore, analogous to head and neck tumors, in cervical carcinomas hypoxia is a major resistance mechanism that impairs outcome and a beneficial effect of hypoxic modification can be expected on theoretical grounds.

Concluding remarks

The addition of carbogen breathing and nicotinamide enhances the effect of accelerated radiotherapy and improves the outcome in a selection of patients with advanced laryngeal cancer. The exogenous marker pimonidazole and several endogenous markers can be applied to predict the response. These markers, combined with clinical characteristics, can select patients for ARCON. Oncological therapies are changing from the 'one treatment fits all' concept to individualized medicine. It is already applied in several types of cancer and this is only the beginning of a new era in cancer treatment.

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8

Summary

Summary

Hypoxia is an important cause of treatment resistance to radiotherapy in head and neck cancer. In this thesis the addition of hypoxic modification to radiotherapy is investigated as a treatment for advanced laryngeal carcinoma. In addition, prognostic and predictive exogenous and endogenous markers are evaluated.

In Chapter 1 an overview is given of the role and pathophysiology of hypoxia, methods of quantification and hypoxia-targeting therapies in head and neck cancer with emphasis on laryngeal cancer. Hypoxia is a negative prognostic factor in head and neck cancer related to tumor aggressiveness and treatment resistance, for the effect of radiotherapy on tumor cells is strongly dependent on the presence of oxygen. Hypoxia is caused by an imbalance between the supply and consumption of oxygen due to rapid growth of a malignant tumor and morphological and functional deformed blood vessels. Tumor cells at a large diffusion distance from a blood vessel will endure chronic hypoxic circumstances, however, due to a temporary disruption of the blood flow, transient hypoxic conditions can exist close to a blood vessel. The result of these mechanisms is a gradual scale of hypoxia within a tumor. The relation of hypoxia with tumor metabolism and proliferation is discussed.

Several methods of measuring hypoxia are available; invasive techniques, exogenous markers and endogenous markers. The prognostic value of several hypoxia- and metabolism-related endogenous markers is reviewed. Finally, several hypoxia-targeting treatment approaches are described, with emphasis on ARCON (Accelerated Radiotherapy plus Carbogen and Nicotinamide).

Chapter 2 describes the results from a randomized trial comparing Accelerated Radiotherapy (AR) with ARCON in laryngeal cancer.

Patients with cT2-4 squamous cell laryngeal cancer were randomized to AR (68 Gy within 36-38 days) or ARCON (AR plus carbogen inhalation and nicotinamide). To limit the risk of laryngeal necrosis, ARCON patients received 64 Gy on the laryngeal cartilage. The primary endpoint was local control. Secondary endpoints were regional control, larynx preservation, toxicity, disease-free survival and overall survival. In a translational side study the hypoxia marker pimonidazole was used to assess the oxygenation status in tumor biopsies.

From 04-2001 to 02-2008, 345 patients were accrued. After a median follow-up of 44 months, local tumor control rate at 5 years was 78% for AR versus 79% for ARCON (p=0.80) with corresponding larynx preservation rates of 84% and 87% (p=0.48). The 5-year regional control was significantly better with ARCON (93%) compared to AR (86%, p=0.04). The improvement in regional control was specifically observed in patients with hypoxic tumors and not in patients with well-oxygenated tumors (100% versus 55% respectively, p=0.01). AR and ARCON produced equal levels of toxicity.

It was concluded that, despite lack of benefit in local tumor control for advanced laryngeal cancers, a significant gain in regional control rate, with equal levels of toxicity, was observed in favor of ARCON. The poor regional control of patients with hypoxic tumors is specifically countered by ARCON treatment.

Chapter 3 introduces an automated analysis of immunohistochemically stained tissue sections to assess the colocalization of tumor-specific prognostic markers. Here, the automated quantitative analysis is used to assess the colocalization of carbonic anhydrase IX (CAIX), a membrane-bound hypoxic marker and Ki-67, a nuclear proliferation marker. Tissue sections of 104 biopsies from 89 patients were stained for CAIX and Ki-67 with diaminobenzidine and haematoxylin counterstain. Image scans of whole tumor sections were recorded and image maps were created with parametric mapping to quantify the markers and assess the colocalization. The fraction of CAIX showed a range of 0–93%. The interobserver correlation and the correlation between manual scores and automated analysis were both very strong (r_s=0.96, p <0.0001, and r_s=0.97, p <0.0001). The labelling index of Ki-67 exhibited a range of 0–42% with less strong but still satisfactory interobserver and manual to automated analysis correlations (r_s=0.90, p <0.0001, and r_s=0.71, p <0.0008). The relative tumor area positive for both markers varied from 0 – 76%. From these results, it was concluded that parametric mapping of immunohistochemically stained tumor sections is a reliable method to quantitatively analyze membrane-bound proteins and assess the colocalization of various tumor markers in different subcellular compartments.

The cellular response of malignant tumors to hypoxia is diverse. Several important endogenous metabolic markers are upregulated under hypoxic conditions. In **Chapter 4** we examined the staining patterns and co-expression of HIF-1α, CAIX, LDH-5, GLUT-1, MCT1 and MCT4 with the exogenous hypoxic cell marker pimonidazole and the association of marker expression with clinicopathological characteristics. Biopsies of 20 advanced head and neck carcinomas were immunohistochemically stained and analyzed. All patients were given the hypoxia marker pimonidazole intravenously 2 h prior to biopsy taking. The tumor area positive for each marker, the colocalization of the different markers and the distribution of the markers in relation to the blood vessels were assessed by semiautomatic quantitative analysis. MCT1 staining was present in hypoxic (pimonidazole stained) as well as non-hypoxic areas in almost equal amounts. MCT1 expression showed a significant overall correlation (r=0.75, p<0.001) and strong spatial relationship with CAIX. LDH-5 showed the strongest correlation with pimonidazole (r=0.66, p=0.002). MCT4 and GLUT-1 demonstrated a typical diffusion-limited hypoxic pattern and showed a high degree of colocalization. Both MCT4 and CAIX showed a higher expression in the primary tumor in node positive patients (p=0.09 both). Therefore, colocalization and staining patterns of metabolic and hypoxia-related proteins provides valuable additional information over single protein analyses and can improve the understanding of their functions and environmental influences.

In **Chapter 5** the prognostic and predictive value of the hypoxia-associated protein CAIX in the ARCON phase III trial is investigated. 261 paraffin embedded tumor biopsies and 79 fresh frozen biopsies from patients entered in the trial were immunohistochemically stained for CAIX. CAIX-fraction and CAIX expression pattern were related to tumor control and patient survival. Low CAIX-fraction was prognostic for worse regional control and overall survival in patients treated with AR. Patients with a low CAIX-fraction treated with ARCON had better regional control and metastasis-free survival compared to AR (RC 97% vs 71%, p<0.01 and MFS 92% vs 69%, p=0.06). Besides the CAIX-fraction, the pattern of CAIX expression was investigated as well. Patients with a perinecrotic CAIX staining pattern had a significantly worse local control, metastasis-free and overall survival compared to patients with a diffuse pattern (65% vs 84%, p=0.01, 70% vs 96%, p<0.01 and 42% vs 71%, p<0.01 respectively), and this could not be improved with ARCON. After multivariate analysis CAIX pattern and N-stage emerged as significant predictors for metastasis-free survival and overall survival. In conclusion, ARCON improves regional control and metastasis-free survival only in patients with low CAIX expression. The different patterns of CAIX expression suggest different mechanisms of upregulation of this glycoprotein and have important prognostic value.

In **Chapter 6** the prognostic and predictive value of Ki-67 was studied, as well as the colocalization with CAIX, applying the method described in **Chapter 3**. Tumor biopsies from 255 patients were immunohistochemically stained for Ki-67 and the hypoxia-related marker CAIX. Labeling index of Ki-67 (Ki-67-LI) and the colocalization with CAIX was related to tumor control and patient survival. On average, tumors from node positive patients had a higher Ki-67-LI compared to node negative patients (median 14% vs 8% p < 0.01). In the cohort treated with AR alone high Ki-67-LI (>10%) was associated with increased regional and distant metastases formation. This was not observed in the patients treated with ARCON. Regional- and distant metastases-free survival were 79% vs 96%, p < 0.01 and 71% vs 88% p = 0.05, for AR and ARCON, respectively. Local control and disease-specific survival were not significantly different between the treatment arms (78% vs 80%, p = 0.91 and 70% vs 76%, p = 0.53). Patients with low Ki-67 expressing tumors had an excellent outcome with accelerated radiotherapy and this could not be further improved with ARCON. Colocalization of Ki-67 with CAIX provided no additional prognostic information. It was concluded that patients with larynx carcinomas with high proliferative activity are at increased risk of regional and distant metastases formation. This risk can be reduced by treatment with ARCON.

9

Summary in dutch
(Nederlandse samenvatting)

Samenvatting

Hypoxie is een belangrijke oorzaak van resistentie voor radiotherapie in hoofd-hals kanker. In dit proefschrift wordt de toevoeging van hypoxie-modificatie aan radiotherapie onderzocht als behandeling voor gevorderde stadia van het larynxcarcinoom. Daarbij wordt gekeken naar prognostische en predictive markers die de juiste patienten voor deze behandeling kunnen selecteren. Onderstaande is een verkorte samenvatting van hoofdstuk 1.

Larynxcarcinoom en radiotherapie

De incidentie van het larynxcarcinoom is ongeveer 700 nieuwe patienten per jaar. Het treft meer mannen dan vrouwen in de verhouding 7:1. Echter, de incidentie onder vrouwen neemt toe, waarschijnlijk ten gevolge van veranderingen in rookgedrag, de belangrijkste etiologische factor. Andere factoren die bijdragen aan het ontstaan van het larynxcarcinoom zijn alcoholgebruik en in mindere mate beroepsblootstelling en humaan papilloma virus (HPV). In de meeste gevallen betreft het een plaveiselcelcarcinoom, in 2/3 van de gevallen supraglottisch gelokaliseerd. De behandeling voor vroege stadia bestaat uit laserchirurgie of radiotherapie met zeer goede lange termijn resultaten. De behandeling van vergevorderde stadia bestaat uit radiotherapie, al of niet gecombineerd met chemotherapie, maar een aanzienlijk deel van de patienten ontwikkelt een locoregionaal recidief of metastasen op afstand. Hypoxie (gebrek aan zuurstof) is een belangrijk resistentie mechanisme wat het slagen van de behandeling in de weg kan staan. Het effect van radiotherapie is namelijk sterk afhankelijk van de aan- of afwezigheid van zuurstof.

Hypoxie in maligne tumoren

In bijna alle maligniteiten speelt hypoxie een belangrijke rol. Het is een negatief prognostische factor, doordat hypoxische tumoren resistenter zijn voor chemo- en radiotherapie en zich een meer agressief tumor phenotype kan ontwikkelen in hypoxische omstandigheden.

Hypoxie ontstaat door een disbalans tussen de aanvoer en het verbruik van zuurstof. In maligne tumoren vormt zich door de snelle groei vaak een chaotische microvasculatuur met slecht functionerende bloedvaten, waardoor de zuurstof toevoer verstoord is. Tumorcellen op grote diffusieafstand van de bloedvaten hebben een chronisch tekort aan zuurstof, maar ook dichtbij de vaten, door een tijdelijk verstoring van de bloedstroom, kan tijdelijk hypoxie voorkomen. Het gevolg is een gradient van hypoxie in het tumorweefsel. Het tumormetabolisme past zich aan aan deze hypoxische omstandigheden door een verhoogde mate van anaerobe glycolyse in plaats van oxidatieve fosforylering. Echter, veel maligne tumoren hebben ook in de aanwezigheid van voldoende zuurstof al een verhoogde mate van glycolyse (Warburg effect), waardoor een verhoogd verbruik van

glucose en vorming van lactaat. Een ander kenmerk van maligne tumoren is de hoge proliferatiesnelheid. Het is gecorreleerd met agressieve tumorkenmerken en leidt tot een slechtere uitkomst voor de patient. Een veelgebruikte endogene marker om proliferatie te meten is Ki-67. Een subgroep van tumorcellen die ondanks de hypoxie wel in staat zijn om te prolifereren, zou de oorzaak kunnen zijn van persisterende of recidiverende ziekte.

Meten van hypoxie

Er zijn verschillende methoden om hypoxie in het tumorweefsel te meten. De focus ligt tegenwoordig op exogene markers, zoals pimonidazole, en een hele range aan endogene tumormarkers. Pimonidazole wordt intraveneus toegediend en bindt aan thiol-bevattende eiwitten in levende hypoxische cellen. Het heeft prognostische waarde in hoofd-hals tumoren, waarbij patiënten met een hoge hypoxische fractie een slechtere locoregionale controle en ziekte-vrije overleving hebben. Het voordeel van de endogene markers is dat ze bepaald kunnen worden op gearchiveerde biopten, zonder voorafgaand infuus, maar er is nog geen marker bekend die herhaaldelijk, robuuste resultaten geeft met betrekking tot prognostische waarde. Een belangrijke potentiele kandidaat is HIF-1 (hypoxia-inducible factor-1), welke de spil is in de cellulaire respons op hypoxie en onder andere proliferatie, apoptose, angiogenese, pH balans en metabolisme reguleert. Het induceert de expressie van carbonic anhydrase IX (CAIX), betrokken bij de pH regulatie, glucose transporters (GLUT's), monocarboxylaat transporters (MCT's) en lactaat dehydrogenase (LDH), allen betrokken bij het celmetabolisme en de glycolyse.

Hypoxie-modificerende behandelingen

Verschillende behandelstrategieën zijn toegepast specifiek gericht tegen hypoxie in tumoren: verhogen van de beschikbaarheid van zuurstof, toxines specifiek gericht op hypoxische cellen en het verminderen van de radioresistentie. ARCON combineert versnelde bestraling met ademen van carbogeen (98% zuurstof) en de toediening van nicotinamide om een zo hoog mogelijke zuurstofspanning in de tumor te verkrijgen. Het heeft in een fase II studie een zeer goed resultaat laten zien in gevorderde stadia hoofd-hals tumoren.

Samenvatting van de navolgende hoofdstukken

Hoofdstuk 2 beschrijft de resultaten van een gerandomiseerde studie die geaccelereerde bestraling (AR) vergelijkt met ARCON in 345 patienten met een cT2-4 larynxcarcinoom. Het primare eindpunt was locale controle, secundaire eindpunten waren regionale controle, larynxpreservatie, toxiciteit, ziekte-vrije overleving en totale overleving. Voor het translationele gedeelte van de studie werd pimonidazole toegediend aan een deel van de patienten om de oxygenatie status van de tumor vast te stellen. Locale controle na 5 jaar was 78% voor AR versus 79% voor ARCON (p = 0.80). De 5-jaars regionale controle was beter met ARCON (93%) vergeleken met AR (86%, p=0.04). Een grotere winst in regionale controle werd behaald in patienten met hypoxische tumoren (100% met ARCON versus 55% met AR, p=0.01). De toxiciteit was gelijk in beide armen. Geconcludeerd werd dat, ondanks het gebrek aan verbeterde locale controle, met ARCON een significante verbetering van regionale controle werd behaald in gevorderde stadia larynxcarcinoom, met name in patienten met hypoxische tumoren.

Om in deze gerandomiseerde studie de prognostische en predictieve waarde van verschillende endogene hypoxie-gerelateerde markers te kunnen onderzoeken wordt in **hoofdstuk 3** een automatische analyse van immunohistochemisch gekleurde weefsel coupes geïntroduceerd, welke geschikt is om de colocalisatie van tumor-specifieke markers te bepalen. In deze studie werd in 104 biopten van 89 patienten de colocalisatie van CAIX en Ki-67 bepaald. Hele coupes werden gescand en met parametric mapping werden numerieke beeldkaarten gegenereerd om de markers te kwantificeren en de colocalisatie te berekenen. De correlatie tussen de handmatige score en automatische analyse was hoog voor CAIX (r_s=0.97, p <0.0001) en Ki-67 (r_s=0.71, p <0.0008). Het relatieve tumoroppervlak positief voor beide markers varieerde van 0 - 76%. Uit deze resultaten werd geconcludeerd dat parametric mapping van immunohistochemisch gekleurde tumor coupes een betrouwbare methode is om kwantitatief membraangebonden eiwitten te analyseren en de colocalisatie van tumor markers in verschillende subcellulaire compartimenten vast te stellen.

Een andere groep van potentiele predictieve markers, die veelvuldig tot expressie komen in maligne tumoren en met name in hypoxische omstandigheden, zijn de metabolisme-gerelateerde endogene markers. In **hoofdstuk 4** werd het expressie patroon en de co-expressie van HIF-1α, CAIX, LDH-5, GLUT-1, MCT1 en MCT4 met pimonidazole onderzocht, en de associatie van deze markers met tumor- en patiëntkarakteristieken in 20 vriesbiopten van gevorderde stadia hoofd-hals tumoren.

MCT1 expressie was in gelijke hoeveelheden aanwezig in zowel hypoxische als niet-hypoxische gebieden en toonde een sterke correlatie in plaats en hoeveelheid met CAIX. LDH liet de sterkste correlatie met pimonidazole zien (r=0.66, p=0.002). Een typisch diffusie-gelimiteerd hypoxisch patroon werd gezien bij MCT4 en GLUT-1, welke een hoge mate van colocalisatie toonden. De expressie van MCT4 en CAIX was hoger in patiënten met lymfekliermetastasen bij diagnose (p=0.09 beide).

Geconcludeerd werd dat colocalisatie en expressie patronen van metabole en hypoxie-gerelateerde eiwitten waardevolle informatie toevoegt aan analyse van enkele eiwitten. Dit kan het begrip en de kennis van de functie van deze eiwitten en de reactie van de tumorcel op de omgeving vergroten.

In **hoofdstuk 5** werd apart de prognostische en predictieve waarde van CAIX in de ARCON fase III studie onderzocht aan de hand van 261 paraffine biopten en 79 vriesbiopten. Patiënten met een lage CAIX-fractie behandeld met AR hadden een slechtere regionale controle en totale overleving dan patiënten met een hoge CAIX-fractie. Met ARCON werd, vergeleken met AR, een hogere regionale controle (97% vs 71%, $p<0.01$) en metastase-vrije overleving (92% vs 69%, $p=0.06$) gezien in patiënten met een lage CAIX-fractie. Het patroon van CAIX expressie (perinecrotisch, diffuus of geen patroon) was een belangrijke prognostische factor, waarbij een slechtere locale controle, metastase-vrije overleving en totale overleving werd gezien bij patiënten met een perinecrotisch expressie patroon. Dit kon niet verbeterd worden met ARCON. CAIX patroon en N-stadium verschenen als enige significante voospellers voor metastase-vrije overleving en totale overleving in multivariate analyse. Geconcludeerd werd dat ARCON een betere regionale controle en metastase-vrij overleving geeft in patiënten met een lage CAIX expressie. Het patroon van CAIX expressie suggereert een verschillend mechanisme van opregulatie en is van prognostische betekenis.

In **hoofdstuk 6** werd de prognostische en predictieve waarde van Ki-67 en de colocalisatie van Ki-67 met CAIX onderzocht met behulp van de methode uit **hoofdstuk 3** in tumor biopten van 255 patiënten. Patiënten met lymfekliermetastasen bij diagnose hadden een hogere Ki-67 labeling index (Ki-67-LI) dan patiënten zonder aangedane lymfeklieren (14% vs 8% $p<0.01$). Ki-67-LI was een slechte prognostische factor in patiënten behandeld met AR alleen. In patiënten met een hoge Ki-67-LI waren de regionale controle en ziekte-vrije overleving hoger met ARCON vergeleken met AR (96% vs 79%, $p<0.01$ and 88% vs 71% $p=0.05$, respectievelijk). Locale controle en ziekte-specifieke overleving verschilden niet significant. Patiënten met een lage Ki-67-LI hadden een zeer goede uitkomst met AR, dit kon niet verder worden verbeterd met ARCON. Colocalisatie van Ki-67 met CAIX gaf geen additionele prognostische informatie. Het blijkt dus dat patiënten met een larynxcarcinoom met een hoge mate van proliferatie meer kans hebben op klier- en afstandsmetastasen. Dit kan worden verminderd door behandeling met ARCON.

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Curriculum Vitae

Saskia Eva Rademakers werd als jongste van 2 dochters geboren op 20 juni 1978 te Bathmen. In 1996 behaalde zij het VWO diploma aan scholengemeenschap de Waerdenborch in Holten, waarna ze begon aan de studie Medische Biologie aan de Universiteit van Utrecht. Drie jaar later, in 1999, verruilde ze haar studie Medische Biologie voor de studie Geneeskunde, eveneens aan de Universiteit van Utrecht. In 2005 behaalde ze haar artsendiploma en was ze 1 jaar werkzaam als ANIOS interne geneeskunde in ziekenhuis Zevenaar en een half jaar als arts op de spoedeisende hulp aldaar.

In april 2007 begon ze aan haar opleiding tot radiotherapeut-oncoloog bij de afdeling radiotherapie van het UMC St. Radboud onder leiding van prof. dr. J.W.H. Leer. Daar werd onder leiding van prof. dr. J.H.A.M. Kaanders en dr. J. Bussink gestart met het onderzoek dat ten grondslag ligt aan het huidige proefschrift. De opleiding werd gecombineerd met het wetenschappelijke onderzoek en de verwachte einddatum van de opleiding is in januari 2015.

Saskia Rademakers woont samen met Geerten Mast en samen hebben zij 2 kinderen.

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