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Hypoxia in larynx carcinomas assessed by pimonidazole binding and the value of CA-IX and vascularity as surrogate markers of hypoxia

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ABSTRACT

Tumour hypoxia as driving force in tumour progression and treatment resistance has been well established. Assessment of oxygenation status of tumours may provide important prognostic information and improve selection of patients for treatment. In this study, a large homogenous group of 103 laryngeal carcinomas has been investigated in the presence of hypoxia by pimonidazole binding and the usefulness of Carbonic anhydrase IX (CA-IX) and vascular parameters as surrogate markers of hypoxia. These parameters are further related to clinical and biological characteristics.

One hundred and three patients with T2–T4 larynx carcinoma were included. They were given the hypoxia marker pimonidazole intravenously (i.v.) 2 h prior to taking a biopsy. Expression of all the parameters was examined by immunohistochemistry, excluding large necrotic areas. Among tumours a large variation in pimonidazole positivity (hypoxic fraction based on pimonidazole, HFpimo) (range 0–19%) and CA-IX expression (hypoxic fraction based on CA-IX staining, HFCA-IX) (range 0–34%) was observed. In 67% of the tumours, hypoxia involved $\geqslant$1% of the viable tumour area. HFpimo and HFCA-IX correlated significantly albeit weak ($p = 0.04$). Both parameters showed weak inverse correlations with the relative vascular area (RVA) ($p = 0.01$). HFpimo was further associated with histopathological grade, with poorly differentiated tumours being more hypoxic. The fraction of the tumour area positive for both pimonidazole and CA-IX correlated significantly with N stage.

From these results, it was concluded that CA-IX and RVA have only limited value for measuring hypoxia and are not as robust as pimonidazole, probably due to the influence of other factors in the microenvironment. A combination of staining patterns of exogenous and endogenous markers might give important additive information about tumour biology and behaviour.

1. Introduction

In cancer development, the role of the tumour microenvironment is increasingly being appreciated. It has been established that most human tumours develop a microenvironment characterised by abnormal tumour vasculature and deficient delivery of oxygen and nutrients.[1] This may consequently act as a selective environment promoting clonal growth of

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genetically altered cells and metabolic alterations, setting the stage for further tumour progression to occur.[2] These adaptive responses are important factors determining the response of tumours to different treatment modalities.

Tumour hypoxia has been associated with worse outcome in various types of cancer, such as carcinomas of the uterine cervix and the head and neck.[3–5] Information on the oxygenation status of tumours may therefore be of great importance for selection and optimisation of treatment. Several methods have been introduced in research laboratories and in the clinic to assess tumour oxygenation.[6] Clinically relevant markers are the 2-nitroimidazoles like pimonidazole.[7] These agents need to be injected intravenously several hours before taking a biopsy and have been demonstrated to be robust markers of hypoxia.[7,8] Their microregional distribution can be visualised by immunohistochemistry. Their use makes it possible to analyse architectural patterns of hypoxia in relation to the vascular network and cellular functions such as proliferation.[7,9] A limitation however, is that they require prior intravenous administration and a large effort has been made to find endogenous markers to assess tumour oxygenation.[10] One of potential clinical relevance is the tumour-associated carbonic anhydrase IX (CA-IX). It belongs to a large family of zinc metalloenzymes which catalyses the reversible hydration of carbon dioxide to carbonic acid and participates in a variety of biological processes including pH homeostasis.[11] Tumour-associated expression of CA-IX is shown to be strongly induced by hypoxia and may serve as contributing factor in acidification of the extracellular environment.[12]

The primary aim of this study was to investigate the presence of hypoxia and the usefulness of CA-IX and vascular parameters as surrogate markers of hypoxia by relating them to pimonidazole binding patterns in a large series of larynx carcinomas. A secondary aim was to study the relation between these parameters and their correlation with clinical and biological tumour characteristics.

2. Patients and methods

2.1. Patients

Between March 2001 and January 2007, 114 patients with laryngeal cancer were included in our hypoxia marker study at the Radboud University Nijmegen Medical Centre. Patients with T2–T4 squamous cell carcinoma of the larynx were included. Approval from the local ethics committee was obtained.

All patients underwent examination under anaesthesia with taking a biopsy and a CT or MRI of the head and neck region including the larynx. For staging of the neck ultrasound with fine needle cytology of enlarged lymph nodes was performed. All patients were discussed in the multidisciplinary head and neck working group for tumour classification and treatment planning.

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

2.2. Immunohistochemical staining for pimonidazole, CA-IX and vessels

From the frozen biopsy material, sections of 5 μm were cut and mounted on poly-L-lysine coated slides and stored at −80 °C until staining for the various markers. Before staining, the sections were fixed for 10 min in acetone at 4 °C and rehydrated in PBS 0.1 mol/L (pH 7.4) (Klinipath, Duiven, The Netherlands). Afterwards sections were incubated in primary antibody diluent (PAD; GeneTex Inc., San Antonio, USA) for 5 min at room temperature. Between all consecutive steps of the staining procedure the sections were rinsed thrice for 2 or 5 min in PBS.

First, the sections were incubated for 30 min at 37 °C with mouse-anti-CA-IX antibody (E. Oosterwijk, Department of Urology, University Medical Centre Nijmegen, Nijmegen, The Netherlands) diluted 1:100 in PAD. The second incubation was for 30 min at 37 °C with goat-anti-mouse (Fab) Cy3 antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted 1:600 in PBS. To block the first mouse monoclonal the sections were incubated for 30 min at 37 °C with donkey-anti-goatF(ab′)$_2$ Cy3 (Jackson Immunoresearch Laboratories) diluted 1:600 in PBS. For detection of pimonidazole, the 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 PAD for 30 min at 37 °C, followed by an incubation period of 30 min at 37 °C with donkey-anti-rabbit Alexa488 (Molecular Probes, Leiden, The Netherlands) antibody diluted 1:400 in PBS. The sections were thoroughly rinsed with PBS and stained for vessels by incubation with the mouse antibody PAL-E (Euro Diagnostica, Arnhem, The Netherlands) diluted 1:6 in PAD.[13] This was followed by incubation for 30 min at 37 °C with chicken-anti-mouse Alexa467 antibody (Molecular Probes) diluted 1:200 in PBS. After the staining procedure, the sections were mounted in fluorostab (ProGen Biotechnik GmbH, Heidelberg, Germany).

2.3. Image acquisition

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, as described earlier.[14] Each tissue section was, according to the appropriate staining protocol, sequentially scanned for the pimonidazole, CA-IX and vessel signals at 100× magnification. 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. In some biopsies diffuse background staining of low intensity was observed. This could be easily separated by choosing an appropriate threshold because there was always a large intensity difference between the very bright marker-specific signal and the faint background.

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

2.4. Analysis

Guided by an H&E staining of a consecutive section, the tumour area of each section was delineated. This area was subsequently used as a mask in further analysis from which non-tumour tissue, large necrotic areas and artefacts were excluded. The hypoxic fractions based on pimonidazole (HFpimo) and CA-IX staining (HFCA-IX) were defined as the tumour area positive for pimonidazole and CA-IX, respectively, relative to the total tumour area. The vascular density (VD) was calculated as the number of vascular structures per square millimetre and the relative vascular area (RVA) was defined as the PAL-E positive area divided by the total tumour area. To determine colocalisation of CA-IX and pimonidazole, the fraction of the total pimonidazole stained area that was also positive for CA-IX was measured. This was calculated by dividing the area positive for both pimonidazole and CA-IX by the total pimonidazole-positive area ($Fpimo_{[CA\text{-}IX]}$).

2.5. Statistical methods

Statistical analyses were done on a Macintosh computer using the SPSS 11.0 (SPSS, Inc, Chicago, IL) and Prism 4.0 (Hearne Scientific software, Dublin, Ireland) software packages. Correlations between parameters, tested as continuous variables, were assessed using the Pearson chi-square test. To determine correlations and differences between these parameters and categorical tumour characteristics (site, T stage, N stage and histolopathological grade) the Spearman correlation coefficient and the One-way ANOVA test for multiple independent samples were applied. In the case of One-way ANOVA test the Bonferroni method was performed for an adjustment of the significance level (basic level $p < 0.05$). $P \leqslant 0.05$ was considered indicative of statistical significance.

3. Results

3.1. Patients

This study included 114 patients from whom biopsies were collected in the operation theatre and immediately stored in liquid nitrogen. Pimonidazole was given to all the patients and none of them had adverse reactions. Eleven patients were excluded from the analysis, six because the histological diagnosis was not squamous cell carcinoma and five because the biopsy contained no or too little tumour tissue. Thus, 103 histologically confirmed squamous cell carcinomas of the larynx were analysed. Of these patients, 29 were women and 74 were men and their ages ranged between 38 and 83 years with a median of 61 years. The primary tumours were localised in the glottic (34) or supraglottic region (69). Table 1 shows patient and tumour characteristics.

Table 1 – Patient and tumour characteristics.

	Number (*N*)
Gender	
Man	74
Woman	29
Tumour site	
Glottic	34
Supraglottic	69
T stage	
T2	31
T3	50
T4	22
N stage	
N0	54
N1	16
N2	33
Histopathological grade	
Well differentiated	6
Moderately differentiated	60
Poorly differentiated	31
Not classified	6

3.2. Hypoxia and vessel staining

All markers gave strong and bright fluorescent signals with little background except in areas of necrosis and occasionally in stromal components of the tumour. Pimonidazole binding was observed in the cytoplasm whereas CA-IX staining was predominantly confined to the cell membrane (Fig. 1). Pimonidazole binding was usually observed at large distances from the blood vessels or near necrosis, whereas CA-IX was already expressed at shorter distances from the vasculature (Fig. 2). Both pimonidazole and CA-IX positivity generally increased with distance from the blood vessels with variable colocalisation between the two signals. Although colocalisation was shown there were also areas where CA-IX expression was found but no pimonidazole and *vice versa* (Fig. 3). In several biopsies both pimonidazole binding and CA-IX expression could be demonstrated; however, not necessarily in the same area of the tumour. In this material 67% and 50% of all biopsies demonstrated pimonidazole and CA-IX positive, $\geqslant$1% of the tumour area, respectively. In 18% and 30%, respectively, the area positive was <1% but >0% and in 15% and 20% there was no pimonidazole and CA-IX at all, respectively. The distribution of HFpimo and HFCA-IX among the biopsies is shown in Fig. 4. Mean, median values and range for all the parameters are shown in Table 2.

3.3. Correlation between pimonidazole, CA-IX and vascular parameters

To investigate the usefulness of endogenous and vascular markers as surrogate markers of hypoxia correlations between pimonidazole binding, expression of CA-IX and vascular parameters were determined. The exogenous hypoxia marker pimonidazole (HFpimo) and the endogenous hypoxia-associated marker CA-IX (HFCA-IX) demonstrated a significant albeit very weak correlation ($r = 0.20$, $p = 0.047$) (Fig. 5). Furthermore, significant inverse correlations could be observed between HFpimo or HFCA-IX and the vascular parameter RVA ($r = -0.25$, $p = 0.011$ and $r = -0.24$, $p = 0.012$, respectively) (Fig. 6). This could however, not be demonstrated for HFCA-IX and

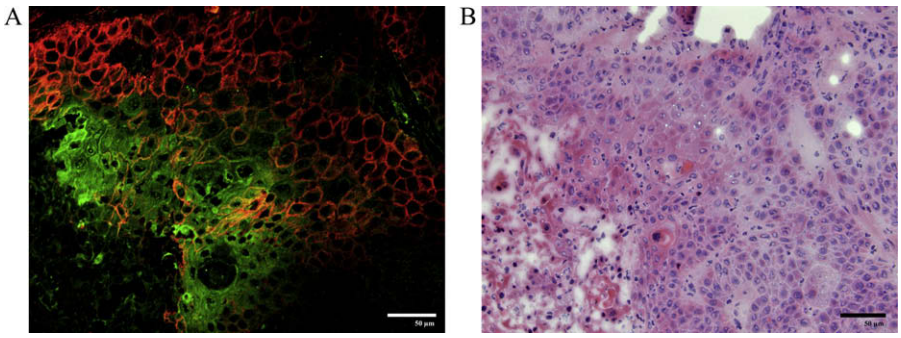

Fig. 1 – Fluorescent and bright field images of squamous cell carcinoma of the larynx. (A) Detailed image showing pimonidazole binding in the cytoplasm and CA-IX staining confined to the cell membrane. *Green*, pimonidazole; *red*, CA-IX; *white*, vessels. (B) Haematoxylin and eosin staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

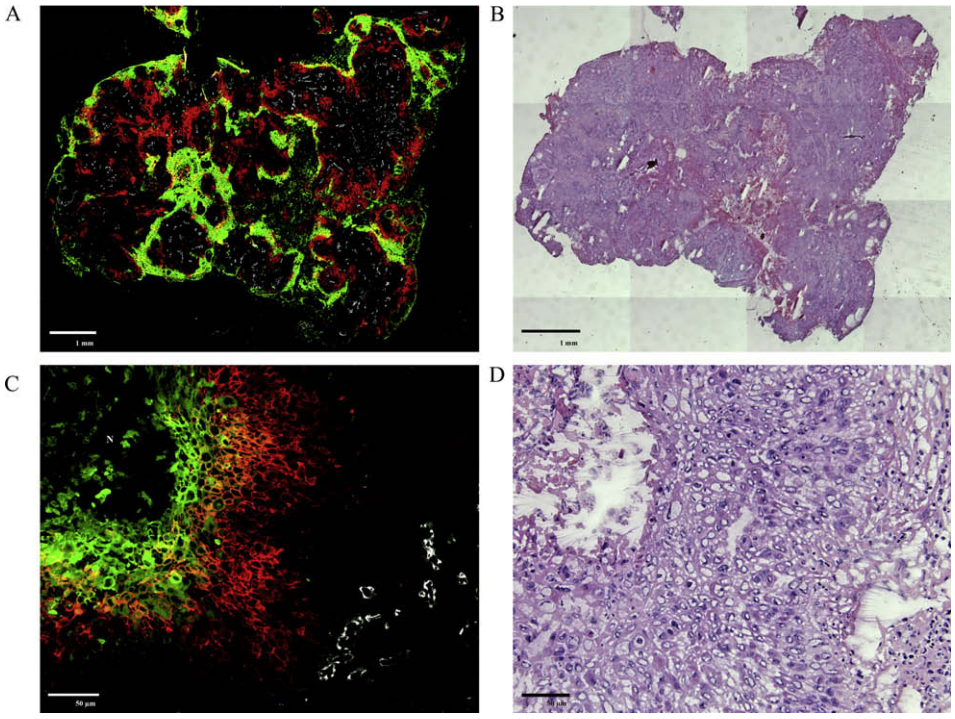

Fig. 2 – Fluorescent and bright field images of squamous cell carcinomas of the larynx. Fluorescent images of squamous cell carcinomas of the larynx showing pimonidazole binding and CA-IX expression. (A) Overview of one biopsy. (B) Haematoxylin and eosin staining. (C) Detailed image showing the relationship of each parameter to the blood vessels and necrosis. (D) Haematoxylin and eosin staining. *Green*, pimonidazole; *red*, CA-IX; *white*, vessels and N, necrosis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

VD, whereas VD showed a trend towards an association with HFpimo ($r = -0.18$, $p = 0.065$).

3.4. *Correlation between microenvironmental parameters and tumour characteristics*

As it is known that microenvironmental tumour characteristics are associated with tumour aggressiveness correlations between these characteristics and clinical and pathological variables were determined. A significant correlation was found between HFpimo and histopathological grade ($r = 0.26$, $p = 0.011$), with poorly differentiated tumours being more hypoxic than moderately differentiated tumours (mean HFpimo 4% versus 2%, $p = 0.015$) (Fig. 7A). No correlations were demonstrated between HFCA-IX, RVA or VD and histopathological grade. Furthermore, HFpimo, HFCA-IX, RVA and VD were independent of tumour site (supraglottic versus glottic) and stage.

Colocalisation of different markers could possibly identify a critical subpopulation contributing to tumour aggressiveness and potentially treatment failure. Between the biopsies large variations in colocalisation between pimonidazole and CA-IX ($Fpimo_{[CA-IX]}$) were observed. An inverse but significant correlation was found between $Fpimo_{[CA-IX]}$ and RVA ($r = -0.22$,

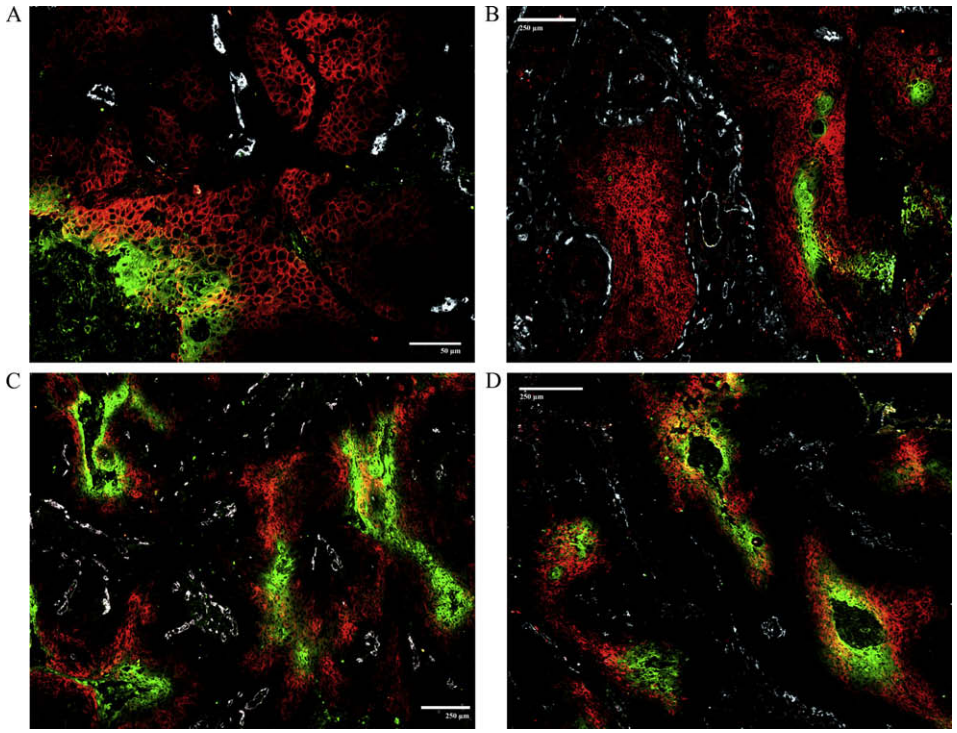

Fig. 3 – Fluorescent images of squamous cell carcinomas of the larynx. Fluorescent images of different larynx carcinomas showing different degrees of colocalisation between pimonidazole and CA-IX. (A) Detailed image showing a large area with CA-IX expression but without pimonidazole binding. (B) No colocalisation. (C) Some colocalisation, also mismatch and (D) High colocalisation. *Green*, pimonidazole; *red*, CA-IX; *yellow*, colocalisation; *white*, vessels. CA-IX expression was also found at short distances from blood vessels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

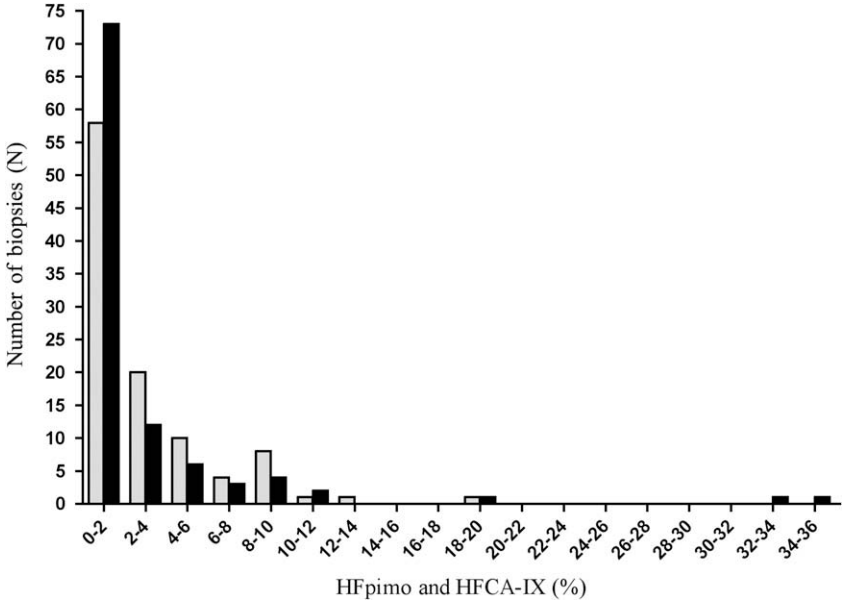

Fig. 4 – Distribution of pimonidazole binding and CA-IX expression. Distribution plot of pimonidazole binding (grey bars) and CA-IX expression (black bars) showing the distribution of HFpimo and HFCA-IX among 103 biopsies.

$p = 0.023$). A significant but weak correlation was also found between $Fpimo_{[CA\text{-}IX]}$ and N stage ($r = 0.27$, $p = 0.045$) (Fig. 7B). It should be noted, however, that there was large overlap between the groups for all these correlations. No other correlations could be found between $Fpimo_{[CA\text{-}IX]}$ and vascular parameters, tumour site, grade and stage.

Table 2 – Values for hypoxic and vascular parameters in biopsies of head and neck squamous cell carcinomas.

	HFpimo (%)	HFCA-IX (%)	VD (N/mm^2)	RVA (%)	Fpimo$_{[CA-IX]}$ (%)
Number	103	103	103	103	103
Mean	2.9	2.5	168.2	1.9	8.9
Median	1.3	0.4	148.2	1.7	1.0
Range	0.0–18.8	0.0–34.4	34.5–876.3	0.08–7.9	0.0–80.2
SD	3.4	5.4	130.3	1.3	16.0

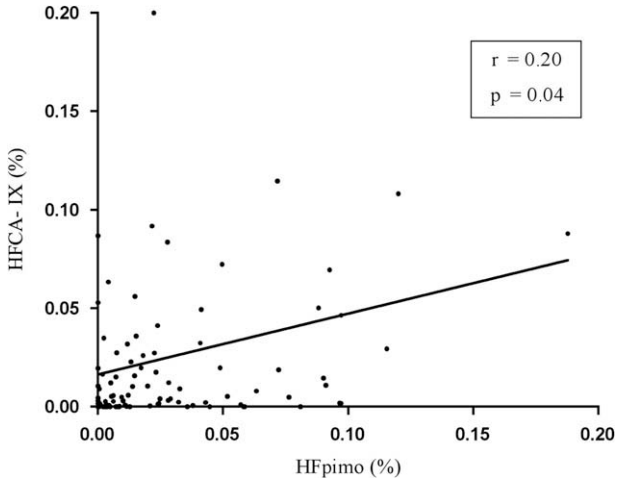

Fig. 5 – Comparison between HFpimo and HFCA-IX. Scatterplot comparing HFpimo with HFCA-IX in 103 squamous cell carcinomas of the larynx. Linear best fit is shown.

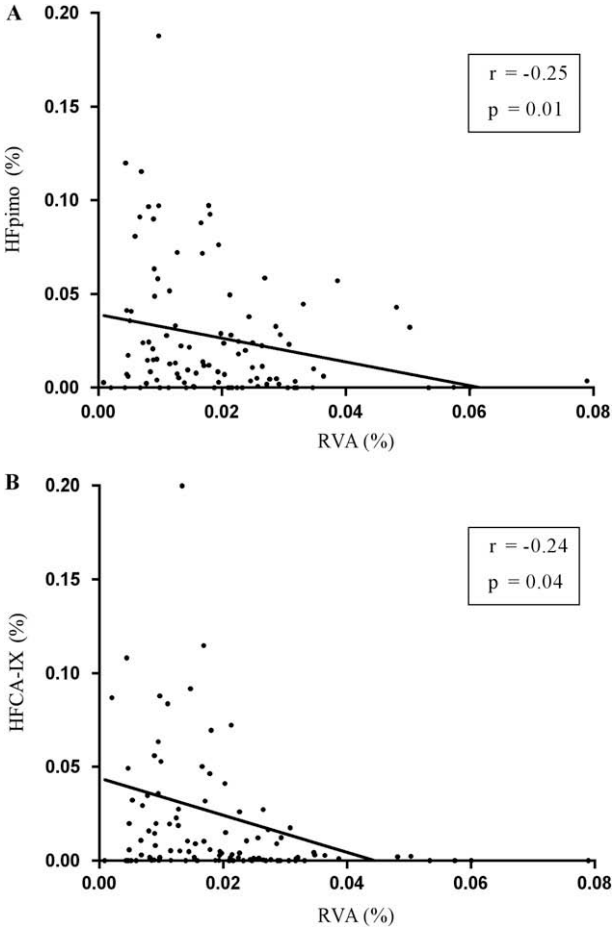

Fig. 6 – Comparison between HFpimo, HFCA-IX and RVA. Scatterplots comparing HFpimo with RVA (A) and HFCA-IX with RVA (B) in 103 squamous cell carcinomas of the larynx. Linear best fit is shown.

4. Discussion

The relevance of hypoxia as driving force in tumour progression and as cause of tumour aggressiveness and treatment resistance has been well established.[3] Assessment of the oxygenation status of tumours will be a valuable contribution to the characterisation of tumours and may provide important information complementary to clinical and pathological characteristics. In conjunction with other parameters reflecting the tumour microenvironment specific profiles may arise that could be predictive for treatment response.

In the present study, a large and homogenous group of laryngeal carcinomas was investigated in the presence of hypoxia, its relationship to known tumour characteristics and the usefulness of endogenous markers as indicators of tumour hypoxia. The pimonidazole binding assay was used as reference assay for determining oxygenation status in all tumour samples. Previous studies have demonstrated the value of pimonidazole as robust marker of hypoxia.[6–8,15] Furthermore, by using the pimonidazole binding assay significant hypoxia has been demonstrated in several tumour types, such as head and neck-, cervical-, colorectal- and prostate carcinomas.[4,16,17] In the present study, 67% and 50% of all biopsies demonstrated pimonidazole and CA-IX positive areas ($\geqslant$1% of the tumour area) respectively, indicative of tumour hypoxia. This is in good agreement with the percentage of pimonidazole and CA-IX positive cases found in other tumour types.[4,17] Both HFpimo and HFCA-IX were independent from clinical tumour characteristics. This was demonstrated before in head and neck carcinomas by Kaanders and colleagues and Eriksen and colleagues.[4,18] At present, in clinical practice tumour stage and site of origin are considered as the main prognostic factors and patients are selected for treatment based on TNM staging. Head and neck carcinomas however, can differ significantly with regard to their microregional phenotype, biological behaviour and response to treatment.[10] Addition of hypoxic or hypoxia-related parameters may supplement TNM staging and improve the selection of patients for certain treatment options or prognostication.

HFpimo was significantly associated with histopathological grade, with poorly differentiated tumours being more

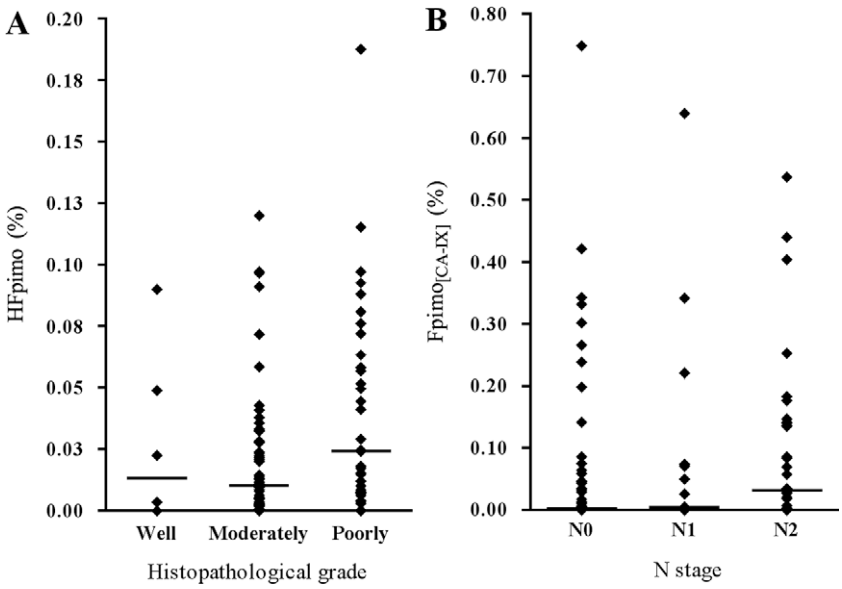

Fig. 7 – Correlation between microenvironmental parameters and tumour characteristics. (A) Distribution of HFpimo between the different groups of histopathological grade (well differentiated, moderately differentiated and poorly differentiated) and (B) Distribution of $Fpimo_{[CA-IX]}$ between the different groups of N stage (N0, N1 and N2). Lines represent the median.

hypoxic than moderately or well-differentiated tumours. Differentiation grade has been shown earlier to be of prognostic significance in cancer of the larynx.[19,20] Furthermore, indications were found that poorly differentiated tumours may metastasise more frequently to cervical lymph nodes.[21] Only a few studies have linked tumour hypoxia to differentiation grade.[22–24] The data are however not conclusive. In carcinomas of the uterine cervix a trend was found to less pimonidazole binding in well-differentiated tumours.[24] The sample sizes were considered to be too small for statistical analyses. To our knowledge this is the first clinical study demonstrating a significant relationship between tumour hypoxia and histopathological grade in laryngeal cancer, possibly reflecting a more aggressive tumour phenotype.

Assessment of tumour hypoxia in patients is usually bound by several technical and logistic difficulties. Planning of intravenous injection of markers a few hours before taking a biopsy can be difficult in the routine practice of a busy clinic. This elicited the search for endogenous markers expressed by the tumour tissue itself. Tumour-associated CA-IX was found to be induced by hypoxia in a broad range of tumour cells.[25] Expression of CA-IX was shown to be induced from oxygen partial pressure (pO_2) levels of ~20 mm Hg and downward. This and previous studies have demonstrated that CA-IX is upregulated at shorter distances from blood vessels, indicating that CA-IX is expressed at higher pO_2 levels than required for reduction of pimonidazole.[4,25] Although in this study a significant correlation between HFpimo and HFCA-IX was found, colocalisation of the two markers was far from complete. Other studies in head and neck cancer and cervical cancer confirm this incongruity between pimonidazole and CA-IX.[4,26] This suggests that CA-IX is probably not as robust as pimonidazole as marker of hypoxia. It also indicates that the two markers identify a distinct population of tumour cells and may thus provide complementary information. Recently, a study by Schrijvers and colleagues[5] demonstrated a relationship between high HIF-1α and CA-IX expression levels and outcome in small glottic carcinomas. The authors explained this by hypoxic radioresistance but it cannot be excluded that HIF-1α and CA-IX are more general indicators of increased tumour aggressiveness. The association of CA-IX expression levels with local control could also suggest that tumour cells under intermediate hypoxia are important in determining response to therapy.[27]

CA-IX upregulation is not only dependent on oxygenation levels but also on the acid–base balance of the tumour tissue. An acidic intracellular pH has been shown to affect cell function, growth and even induce apoptosis. To encounter this acid-load, multiple membrane transport mechanisms such as CA-IX are available on the cell membrane shifting acid into the extracellular environment thereby maintaining a favourable intracellular pH.[12] A low extracellular pH may provide a selective advantage for tumours involving growth and development to a more aggressive phenotype. It was demonstrated that extracellular acidosis facilitates tumour invasiveness and metastasis by promoting degradation of the extracellular matrix and basal membrane.[12,28] Ihnatko and colleagues[29] furthermore postulated that hypoxia induced an increased expression level of CA-IX, which was further elevated by acidosis. It seems plausible that tumour cells under hypoxic and acidic stresses are in increasing need for protection by CA-IX in order to buffer intracellular pH. Svastová and colleagues[30] already demonstrated that hypoxia activated the catalytic activity of CA-IX, resulting in enhanced extracellular acidification. Expression of CA-IX under hypoxia may therefore provide a powerful adaptive advantage during carcinogenesis. This study provides some support for this hypothesis. It was demonstrated that the amount of colocalisation between

pimonidazole and CA-IX correlated significantly with N stage, while both parameters alone did not show any correlation with N stage. This might implicate that cells expressing CA-IX under severe chronic hypoxia have a selective advantage over other tumour cells and that this particular subpopulation might be related to a more aggressive tumour phenotype with increased metastatic potential. Whether the amount of colocalisation between pimonidazole and CA-IX is also related to poor local tumour control and prognosis remains to be elucidated and is currently under investigation.

Not only CA-IX was linked to hypoxia, the relative vascular area (RVA) was also significantly, but inversely, associated with pimonidazole and CA-IX. The more often used parameter vascular density (VD) only showed a trend towards an association. A very limited number of studies, one in human glioma xenografts and another in tumour biopsies from head and neck cancer patients, demonstrated a direct but negative correlation between vasculature and hypoxia. Correlations, however, were always weak.[14,31] A number of studies in head and neck carcinomas demonstrated an association between vascular density and poor local tumour control assuming an important role for tumour hypoxia.[4,32] This study demonstrated a significant association between pimonidazole binding and RVA. This might implicate that RVA is indicative of tumour hypoxia, however the correlation is too weak for RVA to be considered as surrogate marker of hypoxia. It must further be noted that the current study only evaluated the RVA and is not taking into account the functionality of vessels, whether they are perfused or not, if the patient is anaemic and the spatial distribution of blood vessels in relationship to hypoxia.[14] Finally, a limitation of analysing tumour biopsies is that it provides only static information whereas we previously demonstrated in xenograft studies that tumour oxygenation is a dynamic process with changes over time.[15] To monitor the dynamics of tumour hypoxia in the clinic, non-invasive methods such as PET and MRI are required but this will be at the cost of spatial resolution.

In conclusion, a large homogenous group of 103 laryngeal carcinomas was investigated in the presence of hypoxia measured with pimonidazole and endogenous markers as indicators of tumour hypoxia. It was shown that CA-IX and the RVA have only limited value for measuring hypoxia and are not as robust and specific as pimonidazole, probably due to the influence of other microenvironmental factors. The usefulness of endogenous markers as surrogate markers of hypoxia is currently heavily debated. However, when expression patterns of different exogenous as well as endogenous markers are used and combined it might give important additional information about tumour biology and behaviour, which is not reflected in clinical staging. Currently, the predictive role of hypoxia and the tumour microenvironment for treatment outcome is being investigated in a randomised trial employing oxygenation modification in laryngeal cancer.[33]

Conflict of interest statement

None declared.

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