**Clinical radiobiology**

Lack of prognostic and predictive value of CA IX in radiotherapy of squamous cell carcinoma of the head and neck with known modifiable hypoxia: An evaluation of the DAHANCA 5 study

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**Abstract**

**Background and purpose:** CA IX is suggested to be an endogenous marker of hypoxia in tumours like squamous cell carcinomas of the head and neck (HNSCC). The aim of the present study was to investigate whether CA IX served as a prognostic factor for outcome in a large population of HNSCC and if CA IX was able to discriminate the tumours that did benefit from hypoxic modification with nimorazole.

**Materials and methods:** Paraffin-embedded formalin-fixed pre-treatment tumour tissue was available from 320 of the 414 patients treated in the randomized DAHANCA 5 protocol with primary radiotherapy ± the hypoxic radiosensitizer nimorazole. CA IX was measured using immunohistochemistry and results were divided into four groups of CA IX expression: <1%, 1–10%, 10–30% and >30% of the tumour area with positive membrane staining. Locoregional control and disease-specific survival were used as endpoints.

**Results:** Expression of CA IX was not correlated to any of the tumour or patient characteristics investigated. Furthermore, CA IX did not serve as a prognostic marker in the total cohort as well as in the group of 150 patients treated without nimorazole. Finally, no relation was found between the different expression levels of CA IX and the influence of nimorazole when locoregional control or disease-specific survival was used as endpoints.

**Conclusions:** This is to date one of the largest studies of CA IX in HNSCC. The data suggest that CA IX have no prognostic or predictive potential in patients with cancer in the head and neck treated with conventional radiotherapy and concomitant nimorazole.

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Hypoxia has for several decades been acknowledged as a characteristic physiological phenomenon in solid tumours like squamous cell carcinomas of the head and neck (HNSCC) [1] resulting in increased radioreistance and poor prognosis [2,3]. Several different approaches have been made to try to identify hypoxia in tumours [4] and more recently the emphasis has been on the potential use of non-invasive approaches like identification of endogenous markers of hypoxia [5]. One of the more promising markers is carbonic anhydrase 9 (CA IX). CA IX is a member of the carbonic anhydrase family comprising transmembrane enzymes that catalyze the reversible hydration of carbon dioxide to carbonic acid and thereby is involved in the pH homeostasis of cancer cells. In cell line experiments it is seen that CA IX is downstream of HIF-1α and induced in increasing levels, when pO₂ is lowered below 20 mm Hg. CA IX is present in solid tumours as well as in several normal tissues like the gastrointestinal mucosa and helps to maintain a normal intracellular pH under hypoxic conditions resulting in a low extracellular pH thus increasing the breakdown of the extracellular matrix leading to a more malignant phenotype [6,7].

CA IX has primarily been observed in poorly perfused parts of HNSCC and close to necrotic areas [8–10] and in one study CA IX was positively correlated with tumour hypoxia measured by the Eppendorf pO₂ histogram [10]. However, the correlation between CA IX and outcome after radiotherapy has yielded conflicting results. Koukourakis et al. [11] studied CA IX expression in 198 patients from the CHART HNSCC-study. They suggested, that CA IX expression (>10%) served as a negative prognostic factor for loco-regional control and overall survival. In contrast, in a recently published paper from Nijmegen [12] 58 HNSCC

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tumours treated with ARCON were scored for CA IX and positive staining (>25%) correlated with a very favourable loco-regional control whereas no straightforward prognostic value was found in two other studies [13,14]. Thus, at present the prognostic and predictive value of CA IX seems to be debatable. The aim of the present study was to investigate whether CA IX served as a prognostic factor for outcome in a large population of HNSCC and if CA IX was able to discriminate the tumours that did benefit from hypoxic modification with nimorazole.

Materials and methods

Patients

We had access to sufficient paraffin-embedded formalin-fixed pre-treatment tumour tissue from 320 (77%) of the in total 414 patients with HNSCC in the pharynx or supraglottic larynx, enrolled in the randomized DAHANCA 5 protocol. Primary radiotherapy was given with 66–68 Gy in 33–34 fx, 5 fx/wk and the patients were randomized to concomitant treatment with the hypoxic radiosensitizer nimorazole or placebo [15]. Nimorazole is a 5-nitro-imidazole derivative with a relative high affinity for electrons, thereby mimicking the effect of oxygen, thus rendering cells more radiosensitive. There was a complete 5-year follow-up in all patients.

Immunohistochemistry

CA IX was measured using immunohistochemistry (Fig. 1). The optimization involved all steps in the immunohistochemical procedure and the optimal procedure was as follows: slides with formalin fixed paraffin embedded tissue sections of 5 µm were melted for one hour at 60 °C, followed by deparaffination in petroleum and ethanol. Secondly, endogenous peroxidase was blocked in hydrogen peroxide 0.5% (diluted in ethanol) for 20 min. This was followed by boiling in Tris–EDTA glycerol buffer (T–EG buffer), pH 9.0, for 3·5 min in a microwave oven and cooling to room temperature before 10 min embedment in serum-free Protein Block (DAKO X0909). Incubation was performed overnight at 4 °C with the primary monoclonal antibody (clone M75, S. Pastorekova, Bratislava) diluted to 1:1000 in Antibody Diluent (DAKO S0809). On day two,

Fig. 1. Part of tumour sections scored by the four categories (A) <1% of tumour tissue with positive staining for CA IX, (B) 1–10%, (C) 10–30% and (D) >30% of the tumour tissue with positive staining for CA IX.
Envision+ (DAKO K 4001) was applied for 60 min and development was done using diaminobenzidine substrate (DAKO K3468) for 5 min followed by enhancement with 0.5% CuSO4 in 0.05 M Tris-NaCl, pH 7.6, for 10 min. Counterstaining was performed using Mayer’s haematoxylin (1:2 for 2 min) and the slides were mounted with DPX (BDH lab supplies). All intervening washes were done in Tris-PBS-buffer (pH 7.6) with 0.05% Tween 20 (Merck 822184). Negative controls were performed by replacing the primary antibody with Antibody Diluent alone whereas tissue with known positivity for CA IX served as positive control.

Scoring and evaluation
Based on a previous test set of 59 non-consecutive HNSCC, the tumour area with positive CA IX membrane staining was estimated and patients were classified into four pre-defined groups as follows: <1%, 1–10%, 10–30% and >30% of the tumour area with positive CA IX staining. The choice of these cut-points makes it possible to compare the present data with most of the other studies reported in the literature. Pilot studies revealed an acceptable inter-observer variability with a high degree of concordance between sections scored (p < 0.0001), and with a kappa value of 79% regarding determination of the expression into the four categories. For testing the variability of the staining over time, the data-set of 59 non-consecutive slides was stained with more than 1 year of separation and the results of the scoring into the four categories were still highly comparable (p < 0.0001) with only six patients shifting category. A closer look into the CA IX stainings of these six patients revealed that their percent of staining was very close to the predefined cut-points and no really differences were the reason for the shift from one score to another.

Statistics
Statistical evaluations were done using descriptive statistics, Pearson χ2-test, Spearman test for trend (categorical variables) and Kappa-statistics. Univariate survival analyses were performed by the Kaplan–Meier method and compared by the log-rank test, using linear trend for factor levels. Endpoints were 5-year actuarial probability of locoregional control and disease-specific survival. Multivariable analyses were estimated using Cox proportional hazards analysis. All results were considered significant at levels less than 5% (two-sided tests) and odds ratios (OR) were presented with 95% confidence intervals. The SPSS 13.0 statistical package and Sigma Plot 9.0 were used for all the analyses.

Results
Tumour material from 320 patients was available, stained for CA IX and separated by the primary chosen cut-points <1%; 1–10%, 10–30% and >30%. The distribution of CA IX according to tumour and patient characteristics is seen in Table 1. There was no statistical significant association between CA IX scores and tumour/patient variables.

In the total cohort of 320 patients, no prognostic differences were found between the four Ca IX groups when loco-
regional control \((p = 0.9)\) or disease-specific survival \((p = 0.8)\) was used as endpoints (Kaplan–Meier curves not shown). For further studying the prognostic value of CA IX, we looked at the 150 patients treated with placebo in order to eliminate nimorazole as a confounder and in Fig. 2, the Kaplan–Meier curves show no significant influence of CA IX on 5-Y locoregional control in the placebo-group \((n = 150, p = 0.8)\) or in the nimorazole group \((n = 170, p = 0.9)\). These results were reflected as well when disease-specific survival was used as endpoint \((p = 0.4\) and \(p = 0.9\), respectively; Kaplan–Meier curves not shown).

The primary objective in the DAHANCA 5 trial was to investigate the influence of the hypoxic sensitizer, nimorazole, which mimics the effects of oxygen. It therefore seemed reasonable to explore the effect of nimorazole (given the tumour was hypoxic or not) measured by the expression of CA IX. Fig. 3 shows the influence of nimorazole in each of the four almost equally sized CA IX groups. As expected from the original results of the DAHANCA 5 trial [15], patients treated with concomitant nimorazole did better compared to placebo when locoregional control was used as endpoint — although not significant in any of the four groups in the present study \((CA \text{ IX } < 1\%: p = 0.2, \text{ OR } 0.66, \text{ range } 0.29–1.51; CA \text{ IX } 1–10\%: p = 0.4, \text{ OR } 0.65, \text{ range } 0.27–1.58; CA \text{ IX } 10–30\%: 0.2, \text{ OR } 0.51, \text{ range } 0.20–1.30\) and \(CA \text{ IX } >30\%: 0.63, \text{ range } 0.24–1.66\)). Furthermore, not even a trend towards better effect of nimorazole was seen when comparing tumours with low CA IX and increasing expressions of CA IX. These results were confirmed in a Cox multivariate regression analysis using the risk of locoregional failure as endpoint and separated by sensitizer-status: placebo or nimorazole. T-stage \([p = 0.005, \text{ OR } 1.9 (1.2–2.9)]\) and nodal involvement \([p = 0.01, \text{ OR } 1.8 (1.1–3.0)]\) were significant factors in the placebo group whereas only nodal status was a significant factor in the nimorazole group \([p = 0.0008, \text{ OR } 2.3 (1.4–3.8)]\). However, as expected from the univariate analyses the expression of CA IX had no significant influence (overall \(p = 0.8\) in both groups) in any of the subgroups.

The above-mentioned results were closely reflected when disease-specific death was used as endpoint (data not shown): No impact of CA IX expression in any of the sub-groups \((p = 0.8\) in the placebo group and \(0.7\) in the nimorazole group) whereas T- and N-status remained significant with locoregional failure as endpoint.

**Discussion**

The present data are to date the largest study focusing on the importance of CA IX in a HNSCC population treated with primary radiotherapy. The immunohistochemical method seemed to be very robust, estimated from the pilot-studies with a Kappa value of 79% and only discordant in six of 59 tumours even though there was more than one year between the two stainings. Our data showed no correlation between patient- or tumour characteristics and CA IX expression and apparently, no prognostic value of CA IX was demonstrated. In order to avoid confounding, we studied the 150 patients treated with placebo (Fig. 2) and found no impact of CA IX on prognosis either in the placebo-group or in the nimorazole-group. Several other studies have investigated the impact of CA IX expression and outcome after radiotherapy. Some studies have found a poor outcome correlated to high CA IX [11,16] others a favourable outcome [12] whereas some studies [9,13,14] (including ours) did not find any significant difference in outcome between tumours with high and low expression of CA IX. However, it might be difficult to compare these results since the schedule of radiotherapy and the use of hypoxic modification may influence the prognostic value as well as concurrent chemotherapy, different cut-points and methods of immunohistochemistry make it even more difficult to compare data.

![Placebo](image1.png)

![Nimorazole](image2.png)

Fig. 2. The influence of CA IX on locoregional control, when tumours are separated by sensitizer-status: placebo or nimorazole.
In the DAHANCA 5 trial patients were randomized between the hypoxic radiosensitizer nimorazole and placebo concomitant to primary radiotherapy. A recently published study [17] with the same cohort of patients showed, that high levels of plasma osteopontin – another endogenous marker of hypoxia – were able to predict the tumours that did benefit from treatment with nimorazole. Likewise, we assumed that tumours with high expression of CA IX did benefit from concomitant treatment with nimorazole. However, CA IX had no predictive impact on the benefit of nimorazole in any of the four groups and furthermore not even a trend towards better effect of nimorazole was seen when comparing tumours with low CA IX and increasing expressions of CA IX.

These results indicate that CA IX alone is not a specific marker for HNSCC with known modifiable hypoxia which is also supported by the lack of correlation of CA IX and tumour oxygenation status using pO2 needle electrodes in an independent series of 57 HNSCC [18]. Importantly, tumour oxygenation is not the only factor that decreases in a poor microenvironment. Glucose and pH are also often decreased [1] and the interstitial fluid pressure is increased [19]. These factors might also influence the expression of endogenous markers of hypoxia like CA IX [20] and makes the results

Fig. 3. The influence of nimorazole on locoregional control when tumours are separated by CA IX-score.
with such markers even more difficult to interpret, which may explain the discrepancy often seen between the immuno-histochemical staining patterns for CA IX and pimonidazole [21].

The role of CA IX in hypoxia pathways is well described [6,7,10] but probably, no sole endogenous marker is able to predict the hypoxic status of a tumour and we need to build hypoxic profiles as suggested by Nordsmark et al. [18] in order to classify hypoxic tumours more precisely. This may be necessary not only for prognostic reasons, but also for the perspective of using endogenous hypoxic markers as possible molecular targets.

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References