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Original article

Prognostic value of tissue necrosis, hypoxia-related markers and correlation with HPV status in head and neck cancer patients treated with bio- or chemo-radiotherapy

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ABSTRACT

Background and purpose: The aim of the present study was to investigate the role of three hypoxia-related biomarkers in patients with locally advanced head and neck squamous cell carcinoma (HNSCC) treated with concurrent chemoradiotherapy (3-weekly cisplatin) or bioradiotherapy (weekly cetuximab).
Material and methods: In tumor tissue material from 100 patients with known HPV status, we evaluated the extent of tumor necrosis, the expression level of CA-IX and the microvascular density (MVD) measured as the density of CD34+ vascular structures. The correlations between biomarker expressions and clinicopathological characteristics and treatment outcomes were analyzed.
Results: We found a significant correlation of MVD with UICC stage ($p = 0.02$) and T classification ($p = 0.05$), of CA-IX with UICC stage ($p = 0.03$) and N classification ($p = 0.04$) and a significant inverse correlation of MVD with CA-IX expression ($r = -0.22$, $p = 0.03$). Multivariate analysis showed that low MVD combined with high CA IX-expression was a significant independent prognostic factor for worse locoregional control (HR = 2.6, 95%CI 1.1–5.0, $p = 0.02$) in the whole population but not in the p16+ subgroup. Patients treated with CRT had a better LRC than those with BRT independent of MVD or CA-IX expression.
Conclusions: The combination of MVD and CA-IX expression might give additional prognostic information in HNSCC patients with known HPV status.
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Despite the development of modern radiotherapy techniques, combining with chemotherapy or biotherapy, long term survival in locally advanced head and neck squamous cell carcinomas (HNSCC) remains unsatisfactory [1]. In recent years, the role of tumor microenvironment in tumor progression has increasingly been recognized. One of the main characteristics of most tumor microenvironments is the presence of an abnormal tumor vasculature resulting from a process of angiogenesis, responsible from a deficient delivery of oxygen and nutrients unable to cope with the high tumor cell needs [2].

Multiple published studies demonstrated that tumor hypoxia is correlated to poor prognosis in locally advanced HNSCC after radiotherapy [3]. Methods of assessing tumor hypoxia include direct measurements of tumor oxygen tension such as invasive Eppendorf pO2 histograph, functional imaging such as ^{18}F-fluoromisonidazole positron emission tomography (^{18}F-FMISO PET), exogenous biomarkers such as the 2-nitroimidazole-based compounds (characterized by having an NO2 grouping attached to the imidazole ring structure), and endogenous biomarkers. In situ analysis of tissue biomarkers is interesting because it requires no additional invasive procedures other than routine diagnostic biopsy [2].

CA-IX is a member of the carbonic anhydrase family comprising transmembrane enzymes that catalyze the reversible hydration of carbon dioxide to carbonic acid. It is up-regulated by hypoxia and plays a role in pH regulation facilitating acidification of the microenvironment, enhancing cell growth and migration [4]. Although CA-IX over-expression in the tumor is reported to be associated with poor survival rates in esophageal cancer, colorectal cancer and laryngeal cancer [5–7], the role of CA-IX in HNSCC prognosis remains unclear.

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Tumor angiogenesis, critical to support tumor growth, invasion and metastasis, is associated with poor patient outcome and an increased risk of local recurrence and metastasis [8]. Tissue evaluation of angiogenesis usually relies on the evaluation of intratumoral microvascular density using specific vascular markers. CD34 is a single-pass transmembrane protein that is predominantly expressed in small vessel endothelium [9]. While it is generally accepted that angiogenesis is crucial to tumor growth, the clinical prognostic value of MVD measured through the evaluation of CD34+ structures is still being debated.

Tumor necrosis usually reflects the imbalance between tumor growth and its vascular supply required for oxygen and nutrient delivery. Extensive necrosis has been reported to associate with worse survival in renal cancer, colorectal cancer, and lung cancer [10–12].

The purpose of this study was to assess the prognostic value of the three hypoxia-related markers, as well as the possible correlation between these biomarkers and clinical factors in patients with locally advanced HNSCC treated with concurrent chemoradiotherapy (CRT) or bioradiotherapy (BRT).

Methods

Patients and tissues

Patients received total radiation dose of 70Gy, ≥2 cycles of concurrent CDDP or ≥3 cycles of concurrent cetuximab were selected from the former study cohort of 265 patients [13,14]. And 120 patients were matched according to each T and N stages, with a 2:1 ratio of CRT vs. BRT. Then 100 patients who had enough tumor biopsy material available in our institute were included in this study. Formalin ($N = 54$) or formalin-acetic acid-alcohol (FAA) ($N = 46$, patients treated before 2010) fixed, paraffin-embedded pretreatment biopsy material was collected for 100 patients with pathologically-confirmed HNSCC, stage III–IVb according to American Joint Committee on Cancer (AJCC)/International Union for Cancer Control (UICC) TNM classification 2010 treated between June 2006 and October 2012. All pretreatment biopsy slides were reviewed by an experienced pathologist (I.G.). HPV status was determined by p16 immunohistochemistry using CINtec® assay (CINtec P16 INK4A, Ventana, Tucson, AZ, USA). We considered p16 as positive when a nuclear staining was present in >75% of tumor cells; cytoplasmic only staining was considered as negative.

Institutional research ethics board approval was obtained. All patients received either one of the following two treatments: definitive radiotherapy (RT) concomitantly with cisplatin (100 mg/m^2 every 3 weeks on days 1, 22, and 43) or cetuximab (initial loading dose of 400 mg/m^2 one week prior to RT, followed by weekly injection at 250 mg/m^2 during RT). Patients received either three-dimensional conformal radiotherapy (3D-CRT) or intensity-modulated radiotherapy (IMRT) with a total dose of 70 Gy to the gross tumor volume (GTV) in 35 fractions (range 30–35 fractions) at 5 fractions per week, with median treatment time of 49 days (range 39–70 days). A dose of 60 Gy and 50–54 Gy was delivered to the intermediate- and low-risk clinical target volume (CTV). The CTVs were each expanded using 3–5 mm margins to generate planning target volumes (PTV). Patient assessments in follow-up were previously described [14].

Morphological evaluation of tumor necrosis

Three-μm thick sections were cut from paraffin-embedded specimens and stained with hematoxylin and eosin (H&E) for determination of the tumor cell content and for the visual evaluation of the extent of necrosis, defined as the percentage of tissue sample occupied by altered tumor cells showing features of coagulative necrosis (increased eosinophilia, nuclear shrinkage and fragmentation, shadows of preexisting tumor cells) (Fig. 1F).

Immunohistochemistry

Three-μm thick sections were prepared and dehydrated. CA IX expression was detected by using a polyclonal rabbit anti-CA IX antibody (clone NB100-417, Novus Biological, Littleton, CO, USA) at a dilution of 1:250. CD34 expression was determined with a monoclonal mouse anti-CD34 antibody (clone QBEnd/10, NCL-L-END, Novocastra, Newcastle, UK) at a dilution of 1:200. All assessments were performed independently by two pathologists (I. G. and J. A.), blinded to the clinical and follow-up data. Discordances were resolved by consensus review.

For CA-IX evaluation, only membranous staining was scored as positive. The samples were scored as follows: negative, no positive cells; 1+, rare positive tumor cells; 2+, clusters of positive tumor cells, usually adjacent to necrotic areas; 3+, majority of positive tumor cells, usually with a diffuse and widespread distribution (Fig. 1A–D). Two groups were defined as low and high expression: negative and 1+ were regarded as low expression. The staining patterns of 2+ and 3+ were regarded as high expression.

CD34+ structures were counted for evaluating the intra-tumoral microvascular density, as previously described [15]. The first step was a qualitative analysis: presence of positive structures, distribution according to tumor nests and areas of necrosis. Next, quantitative assessment of the density of CD34 + structures was performed. According to international recommendations [16], a single countable micro-vessel was defined as any positively immune-stained endothelial cell or endothelial cell clusters that was clearly separated from adjacent micro-vessels. Structures resulting from the sectioning of the same vessel were considered a single countable micro-vessel. The number of micro-vessels in ten separate areas in a 400× microscopic field (0.2 mm^2 per field) containing the greatest number of micro-vessels was manually counted (Fig. 1E). MVD was expressed as the number of CD34+ structures/mm^2. Digital pictures of all the fields counted in each tumor sample were taken and stored for independent assessments.

Statistics

The associations between CA-IX, CD34 and necrosis expression and the other pretreatment parameters were analyzed using a chi-square test. Spearman's correlation was used to test the relationship between the three marker expressions. Cutoff values for dichotomization of the data were determined for CD34 intensity and necrosis percentage using receiver operating curve analyses (Euclidean distance) for predicting overall survival. Survival rates were calculated with the Kaplan–Meier method. Survival times were defined as the time from the beginning of radiotherapy until either the time of first event or the date that the patient was last known to be alive (censored). Events were death from any cause for OS, death or tumor progression for progression-free survival (PFS), locoregional recurrence for loco-regional control (LRC), and distant metastasis for distant control (DC). Survival curves were compared using the log-rank test. The Cox proportional hazard model was used for multiple regression analysis. Two-sided p values <0.05 were considered to indicate a significant difference. All statistics were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Survival and relapse

The clinical and pathological characteristics of the patients included in this study are summarized in Table 1. The median

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