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Influence of tumor and microenvironment characteristics on diffusion-weighted imaging in oropharyngeal carcinoma: A pilot study

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ABSTRACT

Objectives: Diffusion weighted imaging (DWI) is a frequently performed MRI sequence in cancer patients. While previous studies have shown the clinical value of the apparent diffusion coefficient (ADC) for response prediction and response monitoring, less is known about the biological background of ADC. In the tumor microenvironment, hypoxia and increased proliferation of tumor cells contribute to resistance to (radio-)therapy, while high T-cell influx is related to better prognosis. We investigated the correlation between these three tissue characteristics and ADC in 20 oropharyngeal squamous cell carcinoma patients.

Materials and methods: 20 patients with oropharyngeal squamous cell carcinoma (OPSCC) who underwent 1.5 T MRI, including DWI were included in this pilot study. Corresponding formalin-fixed paraffin-embedded tumor tissues were immunohistochemically analyzed for protein expression of hypoxia-inducible factor 1a (HIF-1a), Ki-67 and CD3. Expression of these markers was correlated with ADC.

Results: ADC negatively correlated with Ki-67 expression ($p = .024$) in tumor cells. There was a significant negative correlation between ADC and CD3-positive cell count ($p = .009$). No correlation was observed between HIF-1a expression and ADC.

Conclusion: This study suggests that ADC reflects characteristics of tumor cells as well as the surrounding microenvironment. Interestingly, high tumor proliferation (a negative prognostic factor) and high T-cell influx (a beneficial prognostic factor) are both associated with a lower ADC. Further studies should be performed to correlate ADC to these histological characteristics in relation to previously known factors that affect ADC, to gain further knowledge on the role of DW-MRI in diagnostics and personalized medicine.

Introduction

In head and neck squamous cell carcinomas (HNSCC) imaging plays a major role in staging, response evaluation and early detection of recurrent disease. Magnetic resonance imaging (MRI) is a modality which is increasingly used, since it provides excellent soft-tissue contrast. Besides conventional anatomical images, additional functional MRI sequences are applied, such as diffusion weighted MRI (DWI). DWI quantifies the restriction of random motion of water molecules in tissues as the apparent diffusion coefficient (ADC) [1,2]. ADC has shown to be useful in differentiating benign from malignant lesions, early treatment response assessment during (chemo)radiation and is promising in prediction of tumor radiosensitivity [3,4].

However, the exact biophysical and biological background of ADC

Abbreviations: HNSCC, head and neck squamous cell carcinomas; OPSCC, oropharyngeal squamous cell carcinoma; DWI, diffusion weighted magnetic resonance imaging; TMA, tissue microarray; CI, confidence interval; ADC, apparent diffusion coefficient

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are not yet fully understood. A recent study showed that ADC is correlated to cellular density and stromal components in tumors. However, it is presumed that multiple tissue characteristics may cause restriction of water molecules [5]. It is hypothesized that perfusion and integrity of cellular membranes also affect ADC but evidence of ADC reflected microanatomical characteristics is sparse [6].

The biological properties of a tumor are not exclusively defined by the neoplastic cells but also by the tumor microenvironment which includes immune cells, endothelial cells and tumor-associated fibroblasts [7]. Neoplastic cells and their microenvironment strongly interact: factors such as tumor hypoxia and subsequent necrosis, or proliferation may contribute to variations in the tumor microenvironment. For example, it has been shown that HPV-associated (HPV-positive) HNSCCs have higher levels of tumor-infiltrating lymphocytes [8]. High lymphocyte count was related to improved survival, independent of HPV-status. Another study showed that HPV-positive tumors have lower ADC-values on DW-MRI, which might reflect differences in microenvironment between HPV-positive and HPV-negative oropharyngeal SCC (OPSCC) [1]. We therefore hypothesized that radiological features of a tumor might not only reflect properties of neoplastic cells but also characteristics within the microenvironment. This may also explain the prognostic value of ADC on survival.

We performed a small, exploratory study, combining data from two previously performed studies, to investigate the correlation between ADC, HPV-status and three characteristics of the tumor and its microenvironment: the presence of T-lymphocytes, tumor hypoxia and tumor proliferation, determined by the CD-3 positive cell count, expression of hypoxia-inducible factor 1alpha (HIF-1a) and expression of the proliferation marker Ki-67, respectively.

Methods and materials

Patient selection

To perform a pilot study on the correlation between tissue characteristics and DWI, two patient databases from previous studies within our institution were combined and resulted in 20 patients who underwent a pretreatment DWI and had tissue available in tissue microarrays (TMAs) [1,9]. While the correlations between histological and DWI data on clinical outcome have been described separately before, the present study describes the correlations between the histology and imaging data. Pre-treatment MRI, including DWI had been performed in a cohort of 75 consecutive patients with a first primary, histopathologically proven HNSCC, treated in our center with (chemo)radiotherapy with curative intent from April 2009 to August 2011. Inclusion criteria were T2, T3 and T4 cancers located in the oral cavity, oropharynx, hypopharynx or larynx. These MRI-scans (including DWI) were part of routine pretreatment imaging.

Tissue from 20 of the 75 the aforementioned patients was available in TMAs created for previous studies [9,10]. Briefly, these were cohorts of 274 OPSCC patients with a first primary OPSCC between 1997 and 2010 in our center. For all studies, follow-up data were obtained at routine outpatient clinic visits. In both studies, leftover material from routine diagnostics was used and obtaining informed consent was not necessary according to laws and 'Best Practice' guidelines in our country. HPV-status was determined by immunohistochemical staining for p16, followed by a molecular HPV-detection test when positive [11,12].

Magnetic resonance imaging protocol

All MRI scans with DWI sequence had been performed for radiotherapy planning purposes. MRIs were acquired on a 1.5 T MRI scanner with 2 surface coils. (Intera NT, Philips Medical Systems, Best, The Netherlands). The MRI protocol consisted of transverse T1-weighted turbo spin echo before and after injection of gadolinium. Transverse and coronal T1-weighted turbo spin echo after gadolinium with spectral presaturation with inversion recovery (SPIR) fat suppression. Transverse and coronal proton density with a short tau inversion recovery (STIR) fat suppression. Included was a transverse diffusion-weighted MRI. Diffusion weighting was achieved by using a single-shot spin-echo planar imaging sequence (TR/TE: 5872 ms:70 ms; EPI factor 51), with a STIR fat suppression with an inversion time of 180 ms and diffusion weighting in three orthogonal directions with b-values of 0, 150, and 800 s/mm^2. Images were acquired with a 112 × 101 matrix, an acceleration factor of 2, a slice thickness of 4 mm, and a slice gap of 0 mm; the number of averages was four. ADC was calculated using all three *b* values. The 3D tumor-volume was manually delineated on the axial slides with a *b* value of 0 s/mm^2 by using the additional information of all other MR images by an experienced head and neck radiologist and an ENT resident in consensus, both having over 5 years of experience with DWI. Evidently necrotic or cystic areas were separately delineated and subtracted from the total tumor volume.

Immunohistochemical analysis

TMAs were constructed and immunohistochemical (IHC) staining was performed as previously described [13,14]. Briefly, representative areas of tumor were marked on hematoxylin and eosin (H&E) stained sections of pre-treatment tumor tissue biopsies, by a dedicated head and neck pathologist. Three 0.6 mm tissue cores per patient were then extracted from the original paraffin block and introduced in the recipient TMA block. Four micrometer sections were stained for Ki-67 and CD-3 protein expression using a Ventana Autostainer (Ventana Medical Systems, Inc, Tucson, USA) and for HIF-1a using a manual staining procedure using the Novolink Kit (Leica Biosystems, Eindhoven, the Netherlands) according to methods described previously [9]. Briefly, slides were deparaffinized and rehydrated, followed by blocking of the endogenous peroxidase activity, antigen retrieval, and incubation with the primary antibody as shown in Table S1. After incubation with the secondary antibody (OV HRP Multimer, Ventana Medical Systems, 8 mins for CD3, Ki-67, Novolink Polymer, 30 mins for HIF-1a), the slides were developed using diaminobenzidine (DAB) and counterstained with hematoxylin, followed by dehydration and coverslipping. On every TMA, normal tonsillar tissues were included as controls. In addition, for every manual staining procedure for HIF-1a, renal cell carcinoma tissue was included as a positive control, and as a negative control by incubation with PBS-BSA instead of the primary antibody.

The stained sections were reviewed by a dedicated head and neck pathologist and an otorhinolaryngology resident in consensus, who were unaware of the clinical data. For CD3, the number of CD3-positive stained cells was manually counted at 400x magnification. Because the TMA-cores were similar in size, normalizing the number of cells for the area was not necessary. For HIF-1a and Ki-67, the percentage of positive stained tumor cells was scored at 200x magnification for each core. Only nuclear staining was considered positive for HIF-1a and Ki67. A mean score of the three cores was calculated for each staining and used for further analyses. Cores were excluded if they could not be evaluated because of folding, when they were missing or when there was less than 5% tumor tissue present in a core.

Statistical analysis

Normality of the variables HIF-1a protein expression, Ki-67 protein expression, CD3 positive staining cells and mean tumor ADC was tested using the Shapiro-Wilk test. In none of these variables, the null-hypothesis of being normally distributed was violated. Correlations between histological data and ADC were analyzed using Pearson correlation with bootstrapping (1000 samples) to provide confidence intervals (CIs). For visual representation of the data, we performed univariate linear regression. P-values below 0.05 were considered statistically significant. All statistical analyses were performed in SPSS