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Original Research Article

Verification of HE-based CTV in laryngeal and hypopharyngeal cancer using pan-cytokeratin

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ABSTRACT

Background: For accurate target definition, we determined margins for the clinical target volume (CTV) for laryngeal and hypopharyngeal cancer in computed tomography (CT, 4.3 mm), magnetic resonance imaging (MR, 6.1 mm) and fluorodeoxyglucose (FDG)-positron emission tomography (PET, 5.2 mm). Previously, we used Hematoxylin-eosin (HE) stained whole-mount sections of total laryngectomy specimens as gold standard to define CTV margins. In the present study, we verified the HE-based tumor delineation with staining for pan-cytokeratin, specific for squamous cell carcinoma.

Methods: Twenty-seven patients with a T3/T4 laryngeal hypopharyngeal tumor were included. From each patient, a total laryngectomy specimen was obtained. Four subsequent 3-mm thick slices containing tumor were selected of which 4-μm thick whole-mount sections were obtained and stained with HE and for pan-cytokeratin CK-AE1/3. Tumors were microscopically delineated on both sections by an experienced head-and-neck pathologist. Tumor delineations were compared using the conformity index (CI) and the distance between both contours.

Results: The CI between HE-based and CK-AE1/3-based tumor delineations was 0.87. The maximum and 95th percentile (p95) extent of the HE-based tumor delineations from the CK-AE1/3-based tumor delineations were 1.7 mm and 0.7 mm, respectively. The maximum and p95 extent of the CK-AE1/3-based tumor delineations from the HE-based tumor delineations was 1.9 mm and 0.8 mm, respectively.

Conclusions: Histopathological assessment of tumor outline on standard HE-stained sections is comparable to microscopic tumor extent based on squamous cell specific pan-cytokeratin staining. Therefore, CTV margins based on HE based tumor contour will be adequate.

Introduction

In radiotherapy, accurate target definition is a crucial step to perform optimal radiation treatment of the tumor [1]. Inaccurate target definition might result in reduced local tumor control or increased side effects in case of overestimation of tumor size [2–4]. Currently, clinical CTV margins lack evidence and are mostly based on clinical experience. Therefore, further validation is needed using histopathology or detailed recurrence localization [5,6].

In current clinical practice, various imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and fluorodeoxyglucose positron-emission tomography (FDG-PET) can be used for gross tumor volume (GTV) delineation [7,8]. Due to the limited resolution of the images and partial volume effects, some microscopic tumorous tissue will not be visible using these imaging modalities. This microscopic tumor tissue, however, needs to be incorporated in the treatment for effective radiotherapy, which is achieved by expansion of the GTV to a clinical target volume (CTV). Expansion of the GTV can be done either by

Abbreviations: HE, hematoxylin-eosin; CTV, clinical target volume; CT, computed tomography; MRI, magnetic resonance imaging; FDG-PET, fluoro-deoxyglucose positron emission tomography; CI, conformity index; p95, 95th percentile; GTV, gross tumor volume; SCC, squamous cell carcinoma; CK-AE1/3, cytokeratin AE1/3 antibodies; TLE, total laryngectomy; HIER, heat-induced epitope retrieval; PBS, phosphate-buffered saline; DAB, diaminobenzidine; TME, tumor microenvironment.

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including anatomical regions with high risk of microscopic spread or by concentric geometric expansion using CTV margins. Geometric expansion might be preferable, as a recent study indicated that this method is less prone to treatment plan variations between different radiotherapy departments [9]. CTV margins have been estimated based on either post hoc evaluation of local recurrences or on examination of the microscopic tumor extent in histopathological specimens [6,10–16]. The literature on the microscopic spread of primary head and neck tumors is sparse [17], but studies by Campbell et al. [10] and Fleury et al. [11] demonstrated that microscopic disease was mainly limited within 5 mm of the GTV defined macroscopically on whole-mount sections. For laryngeal and hypopharyngeal squamous cell carcinomas (SCCs) we concluded, in a previous study, that concentric geometric expansion of the GTV with these CTV margins should be 4–7 mm, dependent on the imaging modality used for GTV delineation [12]. These margins were derived by comparison of the GTVs delineated on CT, MRI and PET with the delineations of the microscopic tumor on whole-mount histopathological sections stained with hematoxylin-eosin (HE). These HE-based delineations were used as gold standard for CTV definition. The assumption was made that all microscopic tumor is visible on the HE stained sections as HE-staining is clinically used to assess excision adequacy by evaluating the presence of tumor at the margins of resection. Furthermore, HE-based tumor delineations show a low interobserver variation among pathologists, which indicates high precision of these delineations [18]. However, the question remained whether HE staining is sensitive enough for detecting all microscopic tumor growth. Therefore, verification of the HE-based tumor delineations is needed using another, more specific staining.

Squamous cells and accordingly SCCs are characterized by the expression of cytokeratins which can be immunohistochemically demonstrated using a pan-cytokeratin staining, such as cytokeratin-AE1/3 (CK-AE1/3), a cocktail of multiple antibodies directed against the epitopes of the most common keratins. Because of the high sensitivity of squamous cells for cytokeratin staining, it is particularly useful in clinical practice to identify or confirm the diagnosis SCC [19]. Therefore, the microscopic tumor visible on HE-stained sections might be intrinsically different from the microscopic tumor visible on sections stained for CK-AE1/3.

In this original study we use pan-cytokeratin CK-AE1/3 staining to investigate whether microscopic tumor delineations essentially differ from HE-staining in inclusion of all microscopic tumor tissue. This addresses the question whether HE based delineations adequately indicate the tumor for CTV margin definition.

Material and methods

Patient and tissue selection

In this study, 24 patients out of 27 patients included from an imaging-validation study were used in the present study [12]. These patients had primary cT3 (N = 4) or cT4 (N = 23) laryngeal or hypopharyngeal squamous cell carcinoma and were imaged with CT, MRI and FDG-PET prior to total laryngectomy (TLE). Tumor stage changed after pathology for one patient from cT3 to pT2, another patient from cT3 to pT4, and another patient from cT4 to pT3. The exclusion criteria for this study were contraindications for CT and for MRI contrast administration and insulin-dependent diabetes mellitus. Three patients were excluded after inclusion, because a tumor biopsy was performed between imaging and surgery, the tumor was fragmented during surgery, and one tumor was too large to fit on the whole-mount slides used for histopathology. The optimization of the specimen preparation process was completed in six patients prior to inclusion of the here reported 27 patients.

From the laryngectomy specimens of these patients, the complete tumor was sliced in axial histological tissue blocks of three millimeter thickness. For each patient, four successive tissue blocks containing tumor and the first cranial and first caudal tissue blocks without tumorous tissue (if available) were selected based on the corresponding HE-stained sections obtained for the imaging validation study [12]. In total, 108 central tissue blocks and 26 cranial and caudal tissue blocks were selected for further analysis, 28 cranial or caudal tissue blocks were missing.

Staining procedure

Microscopic slices were obtained from the selected paraffin-embedded tissue blocks. Per tissue block 10 consecutive 4 μm whole-mount sections were obtained, of which two were used in this study: one stained with HE and another one for CK-AE1/3.

The HE-staining was performed manually according to our clinical staining protocol. The sections were first deparaffinized and rehydrated, then stained with hematoxylin and eosin (Dako, Agilent Technologies, Santa Clara, California) and finally dehydrated and mounted. For immunohistochemical CK-AE1/3 staining, the sections were deparaffinized in xylene and rehydrated in 70% ethanol. Subsequently, the endogenous peroxidase activity was blocked by incubation in 5% solution of 30% hydrogen peroxidase in phosphate buffered saline (PBS) for 15 min. Heat induced epitope retrieval (HIER) was performed in 10 mM sodium citrate buffer (pH 6.0) at 80 °C in a stove for 30 min to reduce cartilage detachment. After HIER, the sections were rinsed with PBS and incubated in the primary antibodies CK-AE1/3 (1:250, Thermo Fisher Scientific Inc., Waltham, Massachusetts) for 60 min. After careful rinsing with PBS, the secondary antibody was applied for 30 min. After applying the secondary antibody and rinsing with PBS, diaminobenzidine (DAB) staining was applied for 10 min. The sections were counterstained with hematoxylin, air-dried and mounted in ClearVue™ (Thermo Fisher Scientific Inc.) (Fig. 1).

Analysis

An experienced head-and-neck pathologist microscopically delineated tumorous tissue on both the HE and CK-AE1/3 stained sections (Fig. 2). The manual delineations with a 0.6-mm thick permanent marker pen were performed independently and separately. The available cranial and caudal sections were checked for presence of tumor deposits by systematic evaluation of these sections using a microscope.

After the first contouring session, the sections were digitized and rigidly registered to each other by selecting manually 3–5 corresponding anatomical landmarks close to or in the tumor on both sections. The delineations were digitized by manually tracing the delineation of the tumor contour using an in-house developed software package [20]. If cartilage was lost during the staining process, resulting in discrepancies between the delineations on both stained sections, the delineations in the cartilage were adapted to either one of the stainings.

The maximum distance between the two delineations was determined. As the goal was to find intrinsic difference between the tumor contour on HE and CK-AE1/3, the larger distances (>2 mm) between the HE-based and the CK-AE1/3 based delineations were re-evaluated. If discrepancies were due to sample and human errors, i.e. tissue displacements, tissue loss or inattentiveness, these delineations were corrected. The revised delineations were used for further analysis.

The conformity of both delineations was measured by the conformity index: