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Effect of radiotherapy on expression of hyaluronan and EGFR and presence of mast cells in squamous cell carcinoma of the head and neck

EVA LINDELL JONSSON[1], KARIN NYLANDER[2], LARS HALLÉN[1] and GÖRAN LAURELL[1]

Departments of [1]Clinical Sciences and [2]Medical Biosciences, Umeå University, SE-901 85 Umeå, Sweden

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Abstract. Head and neck squamous cell carcinoma is a common form of cancer, and despite improvements in treatment during the last decades, survival rates have not significantly increased. There is therefore a need to better understand how these tumours and the adjacent tissues react to radiotherapy, the most common type of treatment for this group of tumours. In order to improve this understanding, the expression of hyaluronan (HA) and epidermal growth factor receptor (EGFR) and the presence of mast cells were mapped before and after radiotherapy using immunohistochemistry. The results showed HA and EGFR to have similar expression patterns in tumour tissue and histologically normal squamous epithelium prior to radiotherapy. Following radiotherapy, EGFR increased in histologically normal epithelium. An increased number of mast cells were also observed as a result of radiotherapy. No expression of EGFR was observed in the connective tissue either prior to or following radiotherapy.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most frequent type of cancer worldwide (1), with more than half a million new cases reported annually (2). The 5-year survival rate is approximately 50%, which is one of the lowest among the more common cancer types. Radiotherapy (RT) remains the major approach to curative treatment despite improvements in chemotherapy schedules (3,4), and it is therefore of interest to study how RT affects potential biomarkers such as those below.

Correspondence to: Dr Eva Lindell Jonsson, Department of Clinical Sciences, Umeå University, Akutvägen, SE-901 85 Umeå, Sweden
E-mail: eva.a.jonsson@ent.umu.se

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Hyaluronan (HA) is a linear disaccharide polymer belonging to the family of glycosaminoglycans, which comprises the major fraction of carbohydrates in the extracellular matrix (ECM). The loose HA matrix provides a favourable environment for mitotic cells and is essential for numerous functions, including cell migration and tissue remodelling during the morphogenesis of organs. HA is also thought to enhance wound healing, tumour growth and metastasis (5). In SCCHN, HA mediates the formation of a complex between the receptor CD44 and the epidermal growth factor receptor (EGFR) (6) which is overexpressed in a large proportion of SCCHN cases (7).

EGFR is a transmembrane protein receptor that plays an essential role in regulating cellular processes such as proliferation, differentiation and survival, and is also central to the maintenance of normal epidermal tissue, where its expression is highly regulated (8,9). When deregulated, EGFR aids in the growth and survival of cancer cells, and is therefore an important target in cancer therapy using monoclonal antibodies (10). EGFR-mediated pathways are involved in the HA/CD44 promotion of chemoresistance in SCCHN (11).

Mast cells are among the first of several immunological cell types migrating to the site of tissue damage, e.g. radio-induced tissue injury. Mast cells cause inflammation by secreting reactive oxygen species, vasoactive molecules, cytokines, chemokines and proteases that remodel the ECM (12). SCCHN tumours, however, have shown a lower number of mast cells than squamous cell carcinoma tumours in other locations (13).

In order to further map the effect of RT in SCCHN we explored the expression of HA and EGFR and presence of mast cells in tumours and adjacent tissue before and after RT.

Materials and methods

Study population. Sixteen patients with SCCHN treated at the Departments of Otolaryngology and Head and Neck Surgery and Oncology, Umeå University Hospital (Umeå, Sweden) between 2001 and 2009 were included. Patients were selected from a larger cohort of patients with the inclusion criteria that they had undergone RT and also had biopsies before and after treatment. Medical records were reviewed and relevant clinical data recorded, including survival, age,

Table I. Patient characteristics.

Patient number	Site	TNM	Differentiation	Irradiation (Gy)	Surgery	Residual tumour	Age at diagnosis (years)	Gender	2-year survival
1	Piriform sinus	T4N3M0	Poor	68	No	No	70	M	No
2	Palatine arch	T2N2cM0	Moderate	68	Yes	No	59	M	Yes
3	Gingiva	T4N1M0	Moderate	68	No	No	51	M	Yes
4	Gingiva	T4N2bM0	Moderate	68	No	No	80	F	Yes
5	Hard palate	T4N0M0	Moderate	68	Yes	No	63	M	Yes
6	Gingiva	T4N2bM0	Moderate	68	No	No	83	M	Yes
7	Bucca	T4N0M0	Poor	68	Yes	Yes	85	F	Yes
8	Tongue	T4N0M0	Poor	68	No	No	48	M	Yes
9	Tongue	T4N2bM0	Poor	68	No	No	43	M	No
10	Gingiva	T4N1M0	Moderate	68	Yes	No	73	F	Yes
11	Tongue	T2N0M0	Poor	68	Yes	No	74	M	Yes
12	Tongue	T4N2cM0	Poor	68	No	No	61	M	Yes
13	Palatine arch	T3N2bM0	Moderate	68	No	No	52	M	Yes
14	Floor of mouth	T4N2bM0	Moderate	68	Yes	No	78	F	No
15	Tonsil	T2N2cM0	Poor	68	Yes	No	53	M	Yes
16	Tongue	T4aN0M0	Moderate	66	No	No	70	M	Yes

tumour site and stage. All patients had at least two years of follow-up (Table I). Informed consent was obtained from the patients prior to the study.

In general, treatment for SCCHN was based on the TNM classification, stage and patient performance. All patients were discussed at a multidisciplinary conference and treatment was given with curative intent.

Patients received RT from a linear accelerator in one daily fraction of 2 Gy 5 days a week, with a mean total dose of 68 Gy.

Seven of the patients received combined modality treatment (preoperative RT followed by surgical resection) and 9 patients single modality RT. The latter 9 patients showed clinical complete response and underwent endoscopic examination at regular intervals to assess the outcome of RT including biopsy of the tumour region for histopathological examination.

Immunohistochemistry. Archival paraffin blocks were cut into 5-μm sections and analysed using immunohistochemistry. Staining for HA was performed according to Hellström *et al* (14). In brief, endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in phosphate-buffered saline (PBS) for 5 min at room temperature. Non-specific binding was then blocked with 1% bovine serum albumin followed by incubation with 100 μl biotinylated HA binding protein (HABP) diluted at a concentration of 1:40 overnight at 4˚C. The Vectastain Elite avidin-biotin complex reagent was then used according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA) together with the diaminobenzidine (DAB) substrate kit (Vector Laboratories). Slides were counterstained with Mayer's haematoxylin. Control slides were incubated with 50 U/ml of Streptomyces hyaluronidase (Sigma, St. Louis, MO, USA), which specifically degrades hyaluronan, for 4 h at 37˚C.

For the detection of EGFR, a monoclonal anti-EGFR antibody (Dako, clone E30) diluted at 1:50 was used. This antibody reacts with an external domain present in the transmembrane 170 kDa protein of both the wildtype EGFR and the EGFRvIII variant. For the detection of mast cells, an antibody recognising tryptase (Dako, clone AA1) diluted at 1:100 was used. The Ventana ES autostainer (Ventana, Tucson, AZ, USA) was used according to the manufacturer's recommendations.

Assessment. All histological samples were reviewed and the grade of differentiation was evaluated by one of the authors (K.N.) who was blinded to the clinical outcome. Two independent scorers (co-authors E.L.J. and K.N.) assessed the slides stained for EGFR and tryptase, and three independent scorers (co-authors E.L.J., K.N. and L.H.) assessed the slides stained for HA.

Staining results for HA were scored in histologically normal epithelium, connective tissue and tumour tissue. Scores representing the percentage of tissue positive for HA were as follows: 0, no staining; 1, 1-25% of the tissue stained; 2, 26-50% stained; 3, 51-75% stained and 4, 76-100% stained. Staining intensity was scored as 0, no staining; 1, weak staining; 2, moderate staining and 3, strong staining. Cellular localisation of HA was further scored as pericellular (PC), intra- and pericellular (IPC), or intracellular (IC), as adapted from Melrose *et al* (15).

EGFR was scored according to Kersemaekers *et al* (16) examining histologically normal epithelium and tumour tissue. The percentage of cells stained as well as the intensity of staining were scored the same way as for HA. The density of tryptase-expressing cells in histologically normal epithelium, connective tissue and tumour was scored in the whole biopsies or surgical specimens and the mean from two independent scorers was calculated.

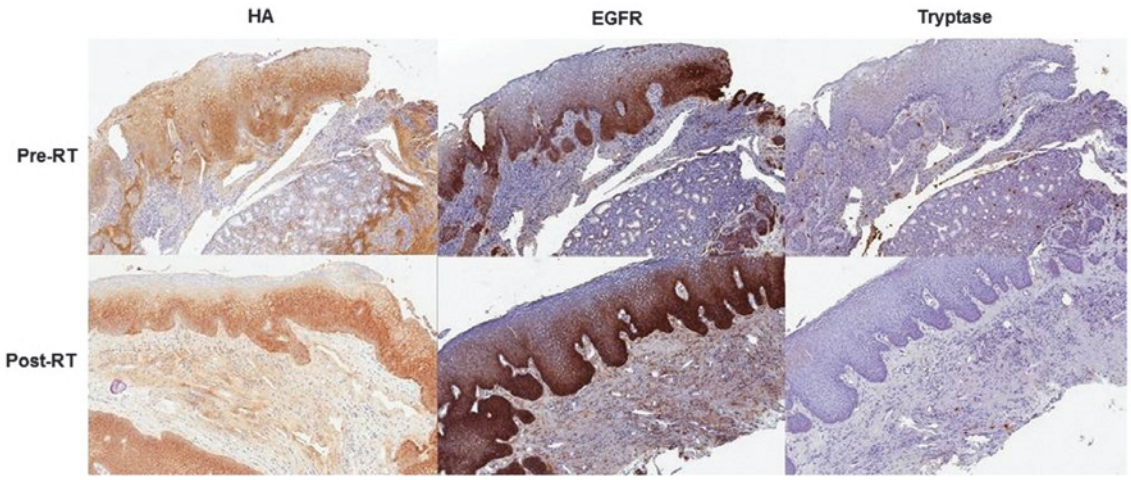

Figure 1. Immunohistochemical analysis of paraffin-embedded tissue pre- and post-radiotherapy (RT) using light microscopy with x10 magnification. Pre-RT HA: HA is mainly localised in the cell layers close to the basal lamina, whereas the more suprabasal layers are negative. HA staining appears to be intracellular in the basal and parabasal layers and pericellular in the more superficial layers. Post-RT HA: Localisation of HA in histologically normal epithelium is not significantly affected by RT, but in the connective tissue an increased expression of HA is observed post-RT. Pre-RT EGFR: Expression of EGFR in histologically normal epithelium is similar to HA expression in the most basal cell layers, and less intense in the more superficial and more differentiated layers. EGFR is found to be intracellularly located in the more basal layers of the epithelium. Post-RT EGFR: The percentage of EGFR-expressing cells in histologically normal epithelium increased following RT. However, no EGFR expression is found in the connective tissue. Pre-RT tryptase: Mast cells are observed throughout the connective tissue, whereas almost no mast cells are observed in normal epithelium. Post-RT tryptase: An increased count of tryptase-expressing cells are observed in histologically normal epithelium post-RT. HA, hyaluronan; EGFR, epidermal growth factor receptor.

Ethical considerations. The study was approved by the University of Umeå Institutional Review Board (registration numbers 01-057 and 03-201).

Results

Fifteen of the 16 patients showed clinical complete response, and the remaining patient showed partial response. The overall 2-year survival was 81% (13 of 16 patients). The median time between RT and surgery was 53 days (range, 40-369) with one outlier who was not initially intended for surgery, while the median time between RT and control biopsy was 75 days (range, 42-305) as the patients undergoing endoscopic examination were not biopsied at fixed intervals, only when a suspected lesion was found during endoscopy.

Immunohistochemistry. Pre-RT biopsies were available for all 16 patients, and were stained for EGFR, HA and tryptase. Two biopsies were excluded from staining with HA and EGFR, and one from staining with tryptase due to a limited amount of tumour tissue for proper evaluation. The amount of connective tissue was limited in many of the pre-RT biopsies but there was sufficient in all samples for evaluation. Seven surgical specimens and nine control biopsies post-RT were available for EGFR, HA and tryptase analyses. A viable tumour was only observed in one specimen following RT treatment.

Expression of HA. In the 12 biopsies with histologically normal epithelium, HA was mainly localised in the cell layers close to the basal lamina, whereas the more suprabasal layers were negative. HA staining was more intracellular (IC) in the basal and parabasal layers and pericellular (PC) in the more superficial layers (Fig. 1). No biopsies demonstrated IC staining, whereas six biopsies demonstrated PC staining. All tumours expressed HA with more intense staining observed in less differentiated tumours; this was similar to HA staining in the basal layers of histologically normal epithelium prior to RT. Two biopsies with poorly differentiated SCC demonstrated IC staining, whereas the remaining 14 tumours demonstrated IPC staining. The localization of HA in histologically normal epithelium was not significantly affected by RT (Fig. 1); the number of cases scored as PC or IPC, and the part of epithelium stained were not affected by RT.

In the connective tissue, increased expression of HA was observed post-RT, and the number of cases where the whole connective tissue expressed HA also increased (Fig. 1). In the case with a remaining viable tumour no change in HA expression was observed.

Expression of EGFR. Expression of EGFR in histologically normal epithelium was similar to HA expression in the most basal cell layers, and less intense in the more superficial and more differentiated layers. EGFR also appeared to be intracellularly located in the more basal layers of the epithelium. All tumours also expressed EGFR, with more intense expression observed in less differentiated tumours. Twelve of the 16 specimens demonstrated the highest proportion of staining (76-100%), and staining was more intense in tumours compared to histologically normal epithelium (Table II). None of the biopsies demonstrated EGFR staining in the connective tissue.

The percentage of EGFR-expressing cells in histologically normal epithelium increased following RT. However, RT did not induce EGFR expression in the connective tissue. In the specimen containing a viable tumour following RT, neither the intensity nor the percentage of EGFR-expressing cells changed but still scored highest in their category.

Presence of mast cells. Mast cells could in general be observed throughout the connective tissue, whereas almost no mast cells

Table II. Changes in the staining patterns induced by radiotherapy (RT).

	Pre-RT			Post-RT		
Tissue	HA	EGFR	Tryptase	HA	EGFR	Tryptase
Histologically normal epithelium	All samples + Basal or parabasal cell layers	All samples + Basal or parabasal cell layers	All samples -	All samples + Basal or parabasal cell layers	All samples + Basal or parabasal cell layers	Few Basal or parabasal cell layers
Connective tissue	All samples +	All samples -	All samples +	All samples +	All samples -	All samples +
Tumour	All samples + Differentiation ↓ → staining ↑	All samples + Differentiation ↓ → staining ↑	All samples +	Remaining tumour +	Remaining tumour +	Remaining tumour +

+, positive staining; -, negative staining; ↑, increase in staining; ↓, decrease in staining; →, leads to.

(mean count, 1.3) were observed in normal epithelium, and five samples were completely devoid. The few mast cells present in the epithelium pre-RT were located basally. Conversely, mast cells were observed infiltrating tumours with a mean count of 2.0 tryptase-expressing cells in pre-RT samples compared to 1.3 in normal epithelium.

The mean count of tryptase-expressing cells in histologically normal epithelium increased from 1.3 to 2.0 post-RT, whereas the mean count in the connective tissue decreased from 18.0 to 11.0 post-RT. The increases and decreases are shown in Table II. In the specimen with viable tumour tissue following RT, tryptase-expressing cells decreased from 10.2 to 3.3.

Discussion

Radiotherapy (RT) remains the main choice of treatment for most patients with SCCHN, either as a single modality treatment or combined with surgery. In order to improve treatment there is a need to better understand how SCCHN and adjacent tissues react to RT, and which side effects the treatment may have. In the present study, the expression of HA and EGFR and the presence of mast cells were studied in biopsies and surgical specimens before and after RT. HA and EGFR showed similar expression patterns before RT both in tumour and histologically normal epithelium. The less differentiated the tumour was, the more intense the staining of both HA and EGFR, a staining pattern similar to that observed in the more basal layers of normal epithelium. This is in accordance with previous results (7,17), and proposed to be due to the role of HA in cell proliferation (18). Earlier studies have shown that staining intensity correlates with the expression of HA (19). Our results confirm the earlier findings of cytoplasmic HA and EGFR, although their role in the cytoplasm is still unclear (20). Membranous staining of EGFR has been shown in clinical studies to correlate with cervical lymph node metastases and survival (21,22). The major ligand for EGFR, EGF, induces migration in connective tissue-derived cells (23). It has been shown that RT induces migration in SCCHN cells *in vitro*, something that is inhibited when EGFR is blocked, suggesting a connection between the mechanisms (24). *In vitro* studies have shown expression of EGFR in connective tissue fibroblasts (25,26), something we could not confirm in the present study either before or after RT. The HA and EGFR-correlated receptor CD44 is also known to be expressed by fibroblasts, and higher HA concentration in cell cultures induces less cell proliferation but more migration in fibroblasts (27).

HA is not, as was earlier believed, just a passive molecule in the ECM but is capable of interacting with ECM macromolecules and cell surface receptors, including CD44 (28). The complex molecular mechanism, including the promotion of CD44/EGFR interaction and EGFR-mediated oncogenic signalling (29), makes it even more significant to analyse a connection between these two markers. Both EGFR and HA overexpression in tumours has been linked to poorer prognosis (30). Our results, in accordance with previous results, showed stronger EGFR staining in tumour tissue compared to normal epithelium (7). We also observed that EGFR staining intensity in normal epithelium increased post-RT, although not to the same level as in tumour tissue pre-RT. The only case with no clinical response/partial response to RT showed the highest EGFR score both before and after RT. This is in accordance with earlier findings that high EGFR expression is correlated with a reduced cellular response to RT (31). Due to its role in cell signalling, EGFR is considered both a predictive marker and a target for cancer therapy. EGFR inhibitors such as C225 (Cetuximab™), a monoclonal antibody to the extracellular domain, have shown radiosensitivity enhancement with amplification of radiation-induced apoptosis in tumour specimens (32). Irradiation is known to induce structural alterations of HA such as degradation (33) and cause alteration of the physical properties; however, to which extent, is yet unknown. HA fragments are further released by most solid tumour cells and activate inflammatory cell signalling, promoting tumour motility (34). In the present study, however, we did not detect any changes in HA expression in normal epithelium or in the one sample with viable tumour cells following RT. A decrease in mast cells was observed post-RT in the connective tissue, but with no correlation to the interval between termination of RT and the time of biopsy or surgical procedure. A minor increase in mast cells in the epithelium was found following RT, and the tumour remaining following RT was also surrounded by

an abundance of mast cells, in accordance with animal studies demonstrating an influx of mast cells during RT (33,34). There is an established correlation between chronic inflammation and cancer (35) and mast cells are known to contribute to pre-malignant progression (36-39). It is known from animal models that mastocytosis in irradiated lung tissue is followed by increased deposition of HA (40,41), which is known to affect the lymphocytic response (42). Our finding that HA was expressed more intensely in the connective tissue stroma post-RT could most probably be viewed as a result of the fibrosis caused by irradiation. This is supported by studies having suggested a possible role for mast cells in fibrosis as a late effect of RT (41).

In conclusion, we have shown RT to have an effect on the expression of HA and EGFR as well as the presence of mast cells in SCCHN tumours. These tumours are, however, known to be heterogeneous (43); therefore, in order to properly evaluate the effect of RT in SCCHN tumours, the tumours should be divided based on subsites in future studies.

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