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A basal-cell-like compartment in head and neck squamous cell carcinomas represents the invasive front of the tumor and is expressing MMP-9

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SUMMARY

Head and neck squamous cell carcinomas (HNSCCs) are the most frequent malignancies of the upper aerodigestive tract. The cancer stem cell (CSC) hypothesis concludes that CSCs constitute the dangerous tumor cell population due to their ability of self-renewal and being associated with relapse of tumor disease, invasiveness and resistance to chemo(radio)therapy. The aim of this study was to look for CSC candidates and expression of MMP-9 that previously was implicated in HNSCC invasiveness.

Immunohistochemical, immunofluorescence and Western blot analysis were performed on HNSCC tumor specimens using antibodies specific for MMP-9, CD44, ALDH1 and CK14. Gelatinolytic activity was assessed by zymography. Pearson correlation analysis was used for statistical comparison.

Immunohistochemical analysis found CD44 and MMP-9 to co-localize in tumor cells at the invasive front. Western blot analysis demonstrated a significant correlation ($p = 0.0047$) between CD44 and MMP-9 in the tested tissues. In addition gelatinolytic activity of HNSCC tissues was found to significantly correlate ($p = 0.0010$) with MMP-9 expression. The $CD44^+$ invasive front of the tumor was also positive for ALDH1 and CK14, all of them being typically expressed by cells in the basal cell layer of normal stratified squamous epithelia that also harbors the epithelial stem cells.

The observations point to a role of a MMP-9 positive basal-cell-like cell layer in the process of HNSCC invasiveness. This compartment likely contains CSCs since it is expressing the putative CSC markers CD44, ALDH1 and CK14. This cell layer therefore should be considered a major therapeutic target in the treatment of head and neck cancer.

Introduction

Each year, more than 400,000 new cases of head and neck squamous cell carcinomas (HNSCCs), representing the clinically most frequent malignancies of the upper aerodigestive tract, are diagnosed world-wide.[1] HNSCC tumors are characterized by early, preferably lymphatic metastatic spread. The prognosis of HNSCC patients drops dramatically if they carry lymph node metastases at the time of diagnosis.

The invasiveness of malignant cells depends on a complex biological program. Matrix metalloproteinases (MMPs) are members of a multigene family of zinc-dependent endopeptidases, and are considered to be key-players in the process of tumor cell invasion.[2] Presently, there exist at least 25 known mammalian MMPs.[3,4] Tumor cells frequently co-express several MMP isoforms, hence, it is not overly surprising that there are many contradictory reports about which specific MMP isoforms are relevant for invasiveness of a respective tumor. It was reported that HNSCC tumors secret high amounts of gelatinases (MMPs 2 and 9).[5] Physiologically, gelatinases are involved in bone development and homeostasis and play a role in the regulation of angiogenesis and vascular remodeling.[3] However, they also play a role in pathophysiological processes such as otitis media and cholesteatoma.[6] It is widely accepted that high expression of MMP-9 positively correlates with the metastatic potential of the primary tumor.[7] In recent years the so-called cancer stem cell (CSC) hypothesis became more and more significant in cancer research. The CSC hypothesis is proposing a model in which only a small subset of tumor cells is able to replicate namely cancer stem cells including their direct progenitor cells. CSCs are not only capable of self-replication but are also able to reproduce the whole phenotype of the original tumor. They are made responsible for relapse of tumor disease, chemo(radio)therapy resistance, invasion and metastasis.[8]

Since CSCs are implicated in the process of invasion and metastasis [9] and were recently described in HNSCCs,[10] the aim of this

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study was to assess the context of CSCs and gelatinolytic MMP activity in this tumor entity.

Materials and methods

Preparation of tissue samples, Western blot analysis and gelatine zymography

Seventeen tissue samples from solid HNSCC tumors were obtained from regularly scheduled surgeries (Table 1). Mucosa, derived from regularly scheduled uvulovelopharyngoplasties (UVPPs) served as a normal control. All tissues were obtained with informed consent of the patients and the study was approved by the local ethics committee. Protein lysates were generated as described earlier.[11] All tissue samples were investigated by gelatine zymography, and Western blot analysis as described elsewhere.[12,13]

Immunohistochemical staining

In short, tissue sections were incubated with anti-MMP-9 (Biozol Diagnostica Vertrieb GmbH, Eching, Germany), anti-CD44 (HCAM (IM7), Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-ALDH1 (ALDH1L1 (YY8), Santa Cruz Biotechnology, Santa Cruz, California, USA) or anti-CK14 (cytokeratin 14 (LL002); Biodesign International; Meridian Life Science, Inc.; Saco, Maine, USA); overnight at 16 °C. For double-labeling experiments the anti-MMP-9 antibody was from Santa Cruz Biotechnology (C20, Santa Cruz, California, USA), whereas the anti-CD44 antibody was as described above. Both primary antibodies were incubated simultaneously over night at 16 °C, washed several times in PBS and detected with species-specific secondary antibodies labeled with either anti-rat-FITC or anti-goat-Texas Red (both Santa Cruz Biotechnology, Santa Cruz, California, USA). Sections were analyzed with a confocal laser scanning microscope (Fluoview, Olympus, Hamburg, Germany) and data were documented as digitized false color images.

Antibodies

The following antibodies were deployed for Western blot analysis: anti-MMP-9 (Biozol Diagnostica Vertrieb GmbH, Eching, Germany), anti-CD44 (HCAM, Santa Cruz Biotechnology, Santa Cruz, California, USA) and anti-β-actin (SIGMA, Saint Louis, Missouri, USA). Secondary HRP-coupled antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). See also above for antibodies used in immunohistochemistry.

Quantification and correlation analysis of MMP-9, CD44 and gelatinolytic activity

To evaluate if the amount of MMP-9 and CD44 protein as well as the level of measured gelatinolytic activity correlate with each other, all bands were quantified by digital densitometry analysis using the MCID Image system (Imaging Research Inc., St. Catherines, Ontario, Canada). For this, the mean density area of the corresponding bands in the Western blot or zymogram was measured and multiplied by the area of the corresponding sampling window on the image. The measured values were normalized to the mean density area of the actin signal that served as an internal loading control. Pearson calculation analysis was performed using the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA, http://www.graphpad.com) to evaluate the level of correlation between the values.

Results

The basal cell layer of normal mucosa is expressing CD44, ALDH1 and CK14

Cells located in the basal cell layer that is known to harbor the stem cells of normal mucosa express the markers CD44 and ALDH1 as well as CK14. Cells of the upper layers loose this expression with higher differentiation. No significant expression of MMP-9 was detected in normal mucosa (Fig. 1a).

Tumor cells that stain positive for basal cell markers are preferentially located at the MMP-9 positive invasive front of the tumor

Immunohistochemistry was implemented to evaluate the presence and localization of putative CSCs in HNSCC tissues. Tissue sections were stained for CD44 since CSCs were previously found to be CD44 positive.[10] As depicted in Fig. 1b, $CD44^+$ HNSCC cells are found predominantly at the invasive front of the tumor that is facing the surrounding stroma. In addition, these tumor cells were also positive for ALDH1, another putative CSC marker (Fig. 1b) [14] that also is expressed in the basal cell layer of normal mucosa

Table 1
Clinical data of tissues used in the study.

Sample #	Localisation/type of tissue	TNM	Grading	Gender	Age (y)
1	Soft palate, reference tissue	–	–	m	44
2a	Laryngeal cancer	T4 N2 M0	G2	m	75
3a	Laryngeal cancer	T3 N1 M0	G2	m	44
4	Laryngeal cancer	T3 N2 M1	G3	m	81
5	Laryngeal cancer	T3 N1 M0	G2	m	68
6	Laryngeal cancer	T3 N0 M0	G2	m	65
7	Oropharyngeal cancer	T2 N0 Mx	G3	m	67
8	Laryngeal cancer	T2 N1 M0	G3	m	52
2b	Laryngeal cancer	T4 N2 M0	G2	m	75
9	Hypopharyngeal cancer	T2 N1 M0	G3	f	71
10	Oropharyngeal cancer	T3 N2 M0	G2	m	52
11	Oropharyngeal cancer	T4 N2 M0	G2	f	69
12	Oropharyngeal cancer	T2 N2 M0	G2	f	53
13	Laryngeal cancer	T3–4 N3 M1	G2	f	69
3b	Laryngeal cancer	T3 N1 M0	G2	m	44
14	Hypopharyngeal cancer	T4 N2 M0	G2	m	52
15	Hypopharyngeal cancer	T4 N3 M0	G3	f	44
16	Hypopharyngeal cancer	T2 N2 M0	G2	m	56

TNM = tumor, node, metastasis (classification according to the guidelines of the International Union Against Cancer). G = grading, G1: well differentiated, G2: moderate differentiated, G3: low differentiated; m = male, f = female.