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Protein signatures associated with tumor cell dissemination in head and neck cancer

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide. Strong prognostic indicators that predict development of distant metastases are the presence and number of lymph node metastases in the neck, and extranodal spread. Recently, it was shown in several studies that also the presence of disseminated tumor cells (DTC) in the bone marrow predicts development of distant metastases. We have investigated whether protein signatures could be detected in primary HNSCC that distinguish tumors that disseminate into the bone marrow from those that do not.

Therefore, DTC-positive and -negative primary HNSCC tumors were analyzed by 2D-DIGE. A signature consisting of 51 differential protein spots was identified upon stratification for bone marrow status, which allowed a correct classification of DTC-positive and DTC-negative HNSCC tumors in 95% of cases, using hierarchical clustering. The most prominent feature within this signature was the down-regulation of CK19 in DTC-positive tumors.

Our data show that tumor cell dissemination to the bone marrow, the onset of hematogenic metastasis, can be deduced from the protein profile in the primary tumor. The highly significant down-regulation of CK19 supports a model of epithelial–mesenchymal transition for tumors that show a high proclivity for hematogenic dissemination.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) arises from the mucosal epithelium of the upper aero-digestive tract. It is the sixth most common type of cancer worldwide [1] and is associated with tobacco use and excessive consumption of alcohol [2,3]. Also the human papillomavirus has been recognized as a risk factor for head and neck cancer [4,5]. Despite improvement in locoregional control for patients with advanced HNSCC by better treatment regimens, overall survival has only marginally increased over the past 3 decades [6], which is frequently caused by distant metastasis [7,8]. Strong prognostic indicators to predict development of distant metastases in HNSCC are the presence and number of metastases in the lymph nodes of the neck [9]. When more than three lymph node metastases (LNM) are identified in the

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neck, the risk of developing distant metastases is almost 50% [10], whereas it is only 7% when the neck is free of LNM [9]. In addition, also extranodal spread is a risk factor for distant disease.

Pantel and Brakenhoff [10] recently proposed a model for carcinomas that spread almost exclusively via lymphatic dissemination. According to this model, disseminated tumor cells (DTC) proliferate in the lymph nodes to form solid metastases, and acquire properties for subsequent hematogenic dissemination. Tumor cells disseminate from these established lymph node metastases through the blood to distant sites, where they can develop into overt metastases. Numerous studies have shown that the bone marrow (BM) is a common homing organ and reservoir for blood-borne DTC, independent of the primary tumor site (e.g. breast, prostate, lung, head-neck and colon cancer) and the pattern of distant metastases [11–14]. Thus far unknown environmental and endogenous factors may promote DTC recirculation from their BM niche into other distant organs such as liver or lungs, where better growth conditions might stimulate proliferation [10,11]. The presence of DTC in bone marrow aspirates, taken from the iliac crest at the time of surgical removal of the primary tumor, can be assessed by highly sensitive and specific immunocytochemistry and PCR-based assays, allowing detection of DTCs at the single cell level in a background of millions of normal cells [11,13].

Currently, most data on the prognostic value of DTC are available for breast cancer, although for several other carcinomas including colorectal, lung and prostate carcinomas an association between the presence of DTC at the time of primary surgery and subsequent metastatic relapse has been reported [11]. Also in HNSCC patients, an association between the presence of cytokeratin-positive cells in the BM and clinical outcome has been observed [15,16]. Partridge et al. [17] reported for HNSCC that detection of DTC in BM corresponded to an increased risk for both local and distant relapse, as well as reduced survival. Recently, Colnot et al. [7] investigated BM aspirates of a larger group of 139 HNSCC patients and demonstrated that the presence of DTC in the bone marrow has prognostic relevance for HNSCC. In particular HNSCC patients diagnosed with multiple LNM at the time of primary surgery could be stratified into low and high-risk subgroups when DTC were detected in BM aspirates [7].

Despite these promising initial data, the used RT-PCR analysis for hLy-6D did not allow to detect all patients at risk for developing distant metastases, as only half of the patients who developed distant metastases were positive in the molecular assay of the bone marrow aspirate [7]. Since the number of detected DTC is generally low (1–2 cells/sample), sampling error may frequently occur. We hypothesized that specific characteristics of the tumor, detectable on the protein level, might facilitate to define the patients at risk for distant metastases. Two-dimensional difference-in-gel electrophoresis (2D-DIGE) is a powerful proteomics platform that enables simultaneous large-scale proteome screening of multiple patient samples, which was applied to analyze the proteomes of 20 primary HNSCC tumors to identify specific marker proteins or protein signatures associated with the presence of DTC in BM.

2. Materials and methods

2.1. Materials

All chemicals were purchased from commercial sources and were of analytical grade. Urea, thiourea, CHAPS, DTT, Tris, immobiline pH gradient (IPG) strips (pH 3–7 non-linear, length 24 cm), IPG buffer (pH3-10 NL), 2D-Quant Kit, 2D-Cleanup Kit, Cy2-, Cy3- and Cy5-CyDyes, DeStreak reagent, DryStrip cover fluid and Cy5-conjugated anti-mouse antibody were purchased from GE Healthcare (Diegem, Belgium). All other antibodies were purchased from DAKO (Glostrup, Denmark). Protease inhibitor cocktail (Complete Mini, EDTA-free) and trypsin (sequencing grade) were obtained from Roche. Flamingo fluorescent gel stain was obtained from Bio-Rad. High purity water obtained from a Milli-Q system (Millipore) was used in all experiments. A Typhoon 9400 imager and DeCyder 2D software (version 6.5.15, EDA module 6.5.15.2) were also purchased from GE Healthcare.

2.2. Tissue processing and microdissection

Fresh-frozen primary tumors were selected from 20 HNSCC patients who consented to enrolment in the study (for clinicopathological characteristics, see Table 1). Based on the presence or absence of DTC in BM aspirates determined by hLy-6D RT-PCR, tumors were stratified as DTC-positive ($n=10$) or DTC-negative ($n=10$). Patient groups were matched for age, gender, site of primary tumor and the presence or absence of LNM. For protein extraction, 30 10 μm sections were prepared from each primary tumor specimen using a Leica CM 1900 cryostat at –25 °C. The sections were collected on Superfrost Plus glass slides, and air-dried at room temperature overnight. For each tumor specimen, a 5 μm section was prepared before and after collecting the 10 μm sections for protein extraction. These separate 5 μm sections were stained with hematoxylin and eosin (H&E) for evaluation by an experienced pathologist, to confirm the presence of invasive carcinoma and to guide microdissection. The 10 μm sections were stained with hematoxylin for 30 s, the day after cryosectioning. After hematoxylin staining, sections were fixed in 70% and 100% ethanol, respectively, and air-dried at room temperature for at least half an hour until microdissection to enrich for tumor cells in the preparation.

Microdissection was performed manually using a Zeiss stereo microscope. The sections were microdissected in such a way that the tumor cell content was at least 80%. Microdissected tumor tissue of all 30 sections per tumor was pooled and transferred to a 0.5 ml microcentrifuge vial with 60 μl of ice-cold extraction buffer containing 30 mM Tris–Cl pH 8.5, 15 mM $MgCl_2$, 7 M urea, 2 M thiourea and protease inhibitor cocktail. At this stage, samples were snap-frozen in liquid nitrogen and stored at –80 °C until further processing.

2.3. Protein extraction from microdissected tissue, quantification and CyDye labeling

After thawing, the extracts were sonicated on ice (6 series of 30 s pulses with 30 s time intervals between the series), and DNA and