Validation of markers for the screening and identification of disseminating tumor cells in lymph nodes and bone marrow

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Introduction

During cancer progression tumor cells acquire the ability to metastasize and this is the most critical event in cancer development. Metastasis is a process with specific, subdivided parts at the molecular level. It involves several sequential steps in a defined cascade and is thus a highly organized process which is non-random and organ-selective [1, 2]. Disseminating tumor cells (DTCs) are potentially metastatic precursor cells, detectable in lymph nodes (LN) and bone marrow (BM). In patients with squamous cell carcinoma (HNSCC) of the head and neck (HNSCC, with ~ 600.000 cases is the sixth most frequent cancer type worldwide) the occurrence of DTCs is associated with a poor prognosis and the development of distant LN metastases in the neck is a highly critical event [2, 3]. The biology of DTCs is still poorly understood partly due to their infrequent occurrence (frequency of ~ 10⁻⁵ – 10⁻⁶) in the tissues examined. The generation of DTCs is thought to be due to rare events taking place during the early and late development stages of the primary tumor [4, 5].

For the screening and identification of DTCs in LN and BM in this study we used the epithelial markers cytokeratin (CK) 5, 14, 18, CD44v6 and EpCAM (epithelial cell adhesion molecule). By using CKs and EpCAM one can clearly distinguish between the non-epithelial cells of LN tissue and BM aspirates and potential DTCs with their origin in the head and neck.

The identification of single DTCs using the markers mentioned allows the detection, isolation, amplification and investigation of the single cell genomes via e.g. metaphase-,

array comparative genomic hybridization (mCGH, aCGH) [6] and single cell sequencing [7]. A detailed molecular investigation of single DTCs will help their characterization and increase our understanding of potential metastatic precursor cells and their global genomic profile.

Material and methods

Study design

LN and BM aspirates from 64 patients with HNSCC were collected (41 males and 23 females; mean age males: 63 years; mean age females: 69 years). Single cell suspensions were prepared and applied on glass slides. The study was approved by the local ethics committee and all patients gave their agreement to the study. Double-immuno-staining was performed (epithelial markers CK 5/14 + CD44v6 (57 patients) and CK18 + EpCAM (50 patients); overlap of 43 patients independent double staining with CK 5/14 + CD44v6 and CK18 + EpCAM) followed by isolation of stained single DTCs via micromanipulator. Genomic DNA of single tumor cells was globally amplified via the adapter-linker-PCR and analyzed via comparative genomic hybridization (CGH) to monitor chromosomal imbalances.

Preparation of lymph node and bone marrow material

Preparation of single cell suspensions from lymph nodes and bone marrow was
performed via Ficoll extraction (GE Healthcare) and sedimentation (bone marrow) or via tissue Medicon preparation (BD Biosciences) and sedimentation (lymph nodes).

**Immunological double staining**

Adhesion slides with $5 \times 10^5$ cells of bone marrow aspirate or lymph node single-cell-solution were permeabilized in 0.5% Triton X-100 (Promega) for 5 minutes. Permeabilized cell solutions were purified in 1× PBS for 3×3 minutes and blocked with 20% human AB-serum for 20 minutes. Cells were incubated for 45 minutes with a primary-antibody-mix against CD44v6 (1:50, mouse, monoclonal, NCL-CD44v6 Leica), cytokeratin 5 (CK5; 1:300, rabbit, monoclonal, NB 110-56916 Novus Biological) and cytokeratin 14 (CK14; 1:300, rabbit, monoclonal, 51054, Abcam) in 10% AB-serum, afterwards slides were washed in 1× PBS for 3×3 minutes. Afterwards incubation for 30 minutes with a secondary-antibody-mix of goat anti-mouse-Alexafluor488 (1:200, A31620 Invitrogen) and goat anti-rabbit-CY3 (1:200, C-2306 Sigma) in 10% AB-serum was performed. Then the slides were purified 3×3 minutes in 1× PBS, subsequently cells were fixed in 1% Paraformaldehyde (PFA) for 4 minutes and finally purified 3×3 minutes in 0.2% 1× Phosphate Buffered Saline with Tween 20 (PBST).

As a negative control for the CD44v6 antibody the X0903 IgG antibody (1:100, rabbit, polyclonal, DAKO) and as negative control for the CK 5/14 antibodies the MOPC-21 IgG1 antibody (1:100, mouse, monoclonal, M5284 Sigma) was used. As a positive control we used the UD-SCC-4 cell line with $1 \times 10^6$ cells per slide (Cytospins).

**Fluorescence microscopy and single cell isolation**

Fluorescence microscopy was performed and isolation of single cells was performed via micromanipulation (glass capillary).

**Results**

Disseminated tumor cells (DTCs) are rare cells and difficult to analyze. In order to detect these cells in lymph nodes and bone marrow of head and neck cancer (HNSCC) patients, we examined lymph nodes and bone marrow aspirates of two groups (at least 50
patients per group) with an immunological staining combination of CK18 and EpCAM as well as CK 5/14 and CD44v6 (Figure 1). We found that the double staining-combination of CK5/14 and CD44v6 resulted in 44% DTC-positive patients (25 of 57 screened patients) compared to 26% DTC-positive patients (13 of 50 screened patients) for the combination of EpCAM and CK18. Between both examined groups there was an overlap of 43 patients who were screened with CK5/14 + CD44v6 and CK18 + EpCAM. In total, 24 LN and 15 BM of 57 patients (CK5/14 and CD44v6) and 13 LN and 5 of BM of 50 patients (CK18 and EpCAM) were DTC-positive in both experimental settings.

The rate of marker positive patients was nearly doubled by using CK5/14 and CD44v6 as markers compared to CK18 and EpCAM. The overlap of 43 patients who were independently stained with both marker-combinations showed that 18 patients were positive using CK5/14 and CD44v6 compared to 13 stained with CK18 and EpCAM. Concerning the number of fluorescent cells, staining with CK5/14 and CD44v6 resulted in an over 3-fold higher number of identified cells (115 single cells) compared to staining with CK18 and EpCAM (35 single cells).

In the overlapping patient group, the use of CK5/14 and CD44v6 as markers for DTCs in HNSCC patients led to considerably higher number of identified DTCs (65 single cells) compared to screenings with CK18 and EpCAM (35 single cells).

A further important aspect was that in the case of double staining with CK18 and EpCAM, the positive cells were only in a few cases double positive (5 of 35 cells 14.29%). Nearly all detected single tumor cells were CK18 positive and EpCAM negative. In the case of CK5/14 and CD44v6 double-staining all detected cells (100%) were positive for the used markers.

**Conclusion**

The study provides new findings from a comparison of two different staining protocols to detect DTCs in lymph nodes and bone marrow aspirates of HNSCC. The screening of DTCs in all lymph nodes and bone marrow samples resulted in a higher number of DTC positive patients with CK5/14 and CD44v6 compared to CK18 and EpCAM double-staining. Further studies are needed to validate the malignant character of the marker-positive cells. This will be possible, since the two double-labeling protocols used allow further comprehensive genomic analysis, e.g. by sequencing or comparative genomic hybridization.

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**References**