Cell line Dependent Expression of EpCAM Influences the Detection of Circulating Tumor Cells With CellSearch

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Objectives: The existence of circulating tumor cells has emerged as an important factor for prognosis and survival. The CellSearch method is the only circulating tumor cell detection method approved by the US Food and Drug Administration for clinical use. It relies on the detection of EpCAM (epithelial cell adhesion molecule) and is approved for colon cancer, breast cancer and prostate cancer. We now investigated whether CellSearch can be used to quantify circulating tumor cells in head and neck squamous cell cancer.

Study Design and Methods: In this study, we investigated the expression of EpCAM in 12 head and neck squamous cell cancer cell lines using Western blot and how this affected their detectability with CellSearch in peripheral blood.

Results: We found a great variation in the expression of EpCAM between our head and neck squamous cell cancer cell lines. This was accompanied by variations in counting efficiency.

Conclusion: We suggest that for reliable quantification of circulating tumor cells in blood from patients with head and neck squamous cell cancer cell, an epitope independent method is preferable.

Key Words: Head and neck squamous cell carcinoma, circulating tumor cells, CellSearch, EpCAM, cytokeratin.

Level of Evidence: NA

INTRODUCTION

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common form of cancer worldwide and there are approximately 650,000 new cases every year.[1] Surgery remains the primary treatment in most cases in combination with radiotherapy, with or without chemotherapy.

As for most solid tumors, HNSCC distant metastases can occur as a result of hematogenous spread as well as lymphatic spread. The existence of hematogenous spread implies that tumor cells are transported within the bloodstream to distant sites where they then establish metastases. The most common cause of treatment failure after surgical removal of HNSCC is, however, local recurrences developing close to the site of the original tumor.[2] Two mechanisms for this have been suggested for histologically tumor-free cases: either the development of new cancers from remaining preneoplastic lesions or through seeding of remaining, but undetectable, tumor cells (minimal residual disease). These cells might be left in the wound after surgery or be blood-born, seeded before or during the operation. In either case, measurement of circulating tumor cells (CTC) might be used to predict the risk of local recurrences or as a way to decide between different treatment options.

We have recently shown that HNSCC cells can become more aggressive when exposed to soluble wound-healing factors.[3,4] This further suggests that CTCs might be the origin of recurrences arising in the surgical wound and that it could be beneficial to determine the amount of CTCs before, during and after surgery so that adjuvant therapy can be tailored to specific patient needs. This further highlights the need for accurate methods for the enumeration of circulating HNSCC cells.

Adequate and reliable identification and quantification of CTCs has recently become available. The CellSearch system (Janssen Diagnostics) is FDA-approved for detection of CTCs in metastatic breast cancer,[5] metastatic colon cancer,[6,7] and metastatic prostate cancer.[8] In breast cancer, recent meta-analyses have determined high clinical validity for metastatic breast cancer[9,10] and prognostic relevance in early breast cancer.[11]

The CellSearch system utilizes antibodies against the epithelial cell adhesion molecule (EpCAM) coupled to magnetic particles for detection of CTCs and the presence of cytokeratin 8, 18, and 19 to confirm the epithelial origin of the cells and thus distinguishing them from

Laryngoscope Investigative Otolaryngology 2: August 2017   Lindgren et al.: EpCAM influences CellSearch results for HNSCC

194

other cells present in whole blood. Only CD45 negative cells are included in order to avoid contamination of leucocytes.

EpCAM is a 38–40 kDa glycoprotein located on the surface of most cells of epithelial origin. It functions both as an adhesion molecule in itself but is also involved in regulating adhesion.[12] It is, however not present in normal squamous stratified epithelia but strongly up-regulated during the neoplastic process of such tissues[13] as well as CTCs from cancers of epithelial origin.[14] In lung cancer, the presence of EpCAM-positive CTCs has been associated with poorer outcomes.[15]

HNSCC tumors are not homogenous in their genotype[16] and there is a great variation in surface antigen expression, ploidy, and growth patterns. Therefore, it is difficult to find specific markers common to all HNSCC cells. This contributes to the great variation in detection levels of CTCs in HNSCC depending on method.[17] Earlier studies have shown that there is a negative correlation between increased number of CTCs and survival but they do not add prognostic or predictive value beyond what is already achieved with TNM status.[18–26] Therefore, simple CTC measurements as performed so far has not proven to give any specific new information that can be used for determining the treatment in the individual case of HNSCC.

Given the described variability of EpCAM expression in HNSCC, we have investigated if and how this could influence the yield in the CellSearch selection process by using HNSCC cell lines with different EpCAM expressions spiked in human whole blood.

MATERIALS AND METHODS

Cell Lines

The cell lines we used were LU-HNSCC-2, LU-HNSCC-3, LU-HNSCC-4, LU-HNSCC-5, LU-HNSCC-6, LU-HNSCC-7, LU-HNSCC-8, LU-HNSCC-16, LU-HNSCC-17, LU-HNSCC-22, LU-HNSCC-24, and LU-HNSCC-25 (abbreviated HN-X where X is the number of the cell line), all of which were established in our laboratory. They were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 units/mL streptomycin sulfate (complete growth medium), under a humidified atmosphere with 5% CO_2.

Western Blot

The cells were grown until approximately 90% confluent. At that point they were lysed in RIPA buffer: 1% Triton X-100, 0.5% sodium deoxy cholate, 0.1% sodium dodecyl sulfate, 150 mmol/L NaCl, 2 mmol/L Na_3VO_4, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, complete protease inhibitors (Roche Applied Science, Basel, Switzerland), 1 mmol/L ethylenediamine tetra-acetic acid (EDTA) and 50 mmol/L Tris–HCl, pH 7.4. Protein concentration was analyzed using the micro bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin (BSA) as standard. Equal amounts of protein were separated on 4–12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The proteins were blotted to polyvinylidene fluoride membranes and incubated with primary epithelial cell adhesion and activating molecule (EpCAM) rabbit monoclonal antibody D1B3 or cytokeratin 8/18 (C51) and cytokeratin 19

(BA17) mouse monoclonal antibodies, all from Cell Signaling Technology (Danvers, MA, USA). Antibody binding was detected using an anti-rabbit immunoglobulin (IgG) horseradish peroxidase-linked antibody (Cell Signaling Technology) and the ECL Prime detection system from GE Healthcare (Fairfield, CT, USA). The staining intensity was determined using a FluorChem FC2 imaging system and the AlphaView software package (Cell Biosciences, Santa Clara, CA, USA). Loading control was performed using Coomassie R-350 staining.[27]

Addition of Cells to Human Whole Blood

Blood samples were collected from a volunteer in Cellsave tubes (Janssen Diagnostics, Raritan, NJ, USA) one day before addition of cells. The tubes were weighed before and after sampling for calculation of blood volume and stored at room temperature.

The cells were grown until approximately 90% confluent, then trypsinized for exactly 7 min and counted four times or more in a Countess cell counter (Invitrogen, Carlsbad, CA, USA). After dilution to 20,000 cells/mL in complete medium, 10 μL (thus containing a calculated 200 cells) was added to each blood sample. CellSearch counting was performed within 3 hours from addition of cells.

Enumeration and Collection of CTCs

The CellSearch® system (Janssen Diagnostics, Raritan, NJ, USA) was used for enumeration of CTCs from 7.5 ml of whole blood applying immuno-magnetic capturing of CTCs as described previously.[14,28] In short, using ferrofluid nanoparticles with antibodies against epithelial cell adhesion molecule (EpCAM), CTCs were magnetically separated from the bulk of other cells in the blood. Subsequent staining for cytokeratins 8, 18, and 19 (CK) was done within the system with a phycoerythrin (PE) coupled antibody against CK for positive identification of CTCs. The enriched cells were also stained for CD45 with an APC-coupled antibody for negative identification of contaminating leukocytes, and with DAPI for identification of nucleated cells. CTCs were defined by specific morphologic criteria together with the immune-phenotype CK+/DAPI+/CD45-in the CellTracks Analyzer II fluorescent microscope.

The number of cells in 10 mL was calculated from the CellSearch count and the total blood volume in each tube and reported as a percentage yield in relation to the calculated 200 cells added per tube.

RESULTS

EpCAM Expression

The total expression of EpCAM protein was measured in 12 HNSCC cell lines (Fig. 1). In one cell line (HN-3) there was no detectable expression. The other cell lines all expressed EpCAM, with the highest expression being 15 times higher than the lowest (HN-7 compared with HN-25). Five of the cell lines had an expression well above (>150%) of the average while the rest had an expression below the average (<85%).

Enumeration of Tumor Cells in Whole Blood

Eleven cell lines with a range of EpCAM expressions were spiked into whole blood and subsequently analyzed by the CellSearch method. The yield in the counting process ranged from 58 to 106% (Fig. 2).
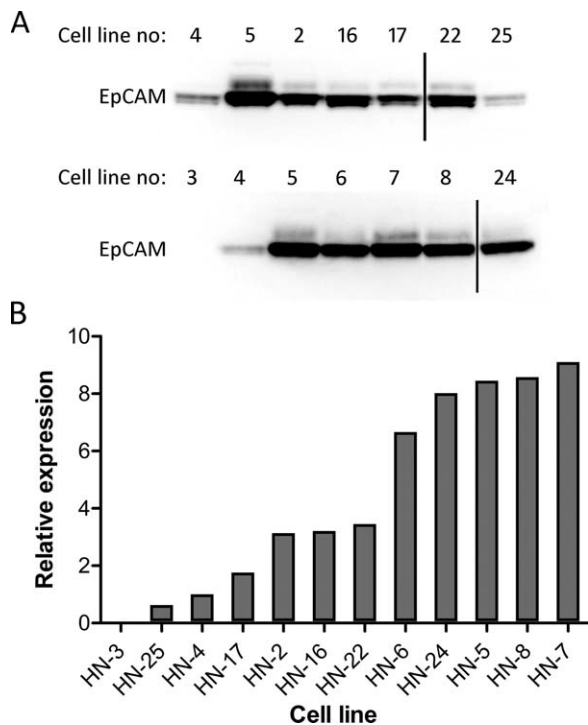
Fig. 1. The expression of EpCAM protein in head and neck squamous cell carcinoma cell lines. A. The cell lines were lysed and analyzed by Western blotting. B. The Western blot was quantified in relative measures (N = 2).

The cell lines with high EpCAM expression had yields above 95%, except HN-24 for which 73% of the cells were counted (mean 95%). The cell lines with low expression all had a yield well below 100% (range 52–74%, mean 63%). When comparing the high expressing with the low expressing cell lines there was a statistically significant difference in the yield percentages (P = .0087) (Fig. 3).

Expression of Cytokeratins

Cytokeratin 8, 18, and 19 are used in the Cell-Search process to detect the tumor cells after the
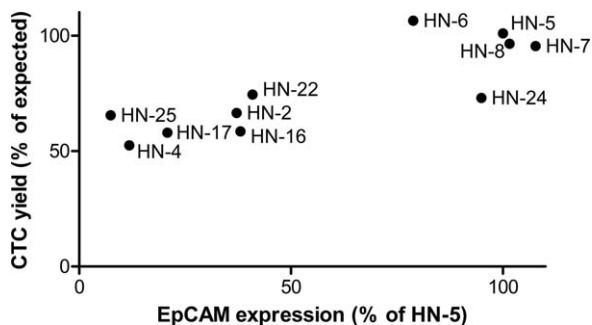

Fig. 2. Efficiency in CTC counting of head and neck squamous cell carcinoma cells spiked in whole blood. Cells were trypsinized, counted and added to whole blood from a healthy donor. The cancer cells were then enumerated by the CellSearch method as outlined in the Method section (N = 1). The percent yield is plotted against the EpCAM expression as measured by Western blotting.
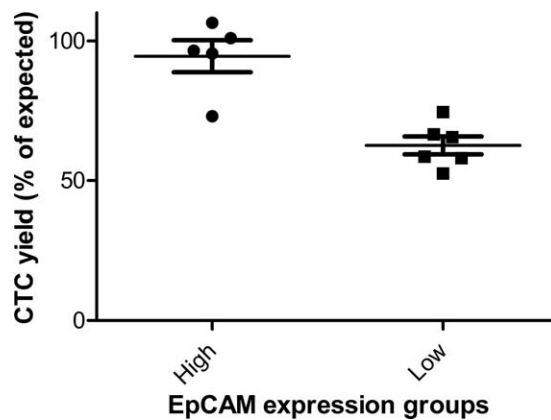

Fig. 3. Group analysis comparing the counting efficiency of high (>mean) and low (<mean) EpCAM expressing cell lines. The difference between the groups was statistically significant (P = .0087, Mann-Whitney U-test).

selection process. As varying expression of these proteins also might affect the final yield we analyzed the expression in the cell lines used in the CellSearch counting (Fig. 4). Three cell lines (HN-4, HN-7 and HN-25 had a notably lower expression of these cytokeratins compared with the other cell lines. HN-5 expressed only cytokeratin 8, while the others expressed mainly cytokeratin 18. The expression of cytokeratin 19 was low in all cell lines. There were no significant differences in yield between high expressers (above mean) and low expressers (below mean) of cytokeratin 8, 18 or the combination of 8 and 18 (P = .073, 0.93, and 0.33, respectively) (data not shown). Interestingly, the cell line with the lowest expression of cytokeratin, HN-4, was the only one for
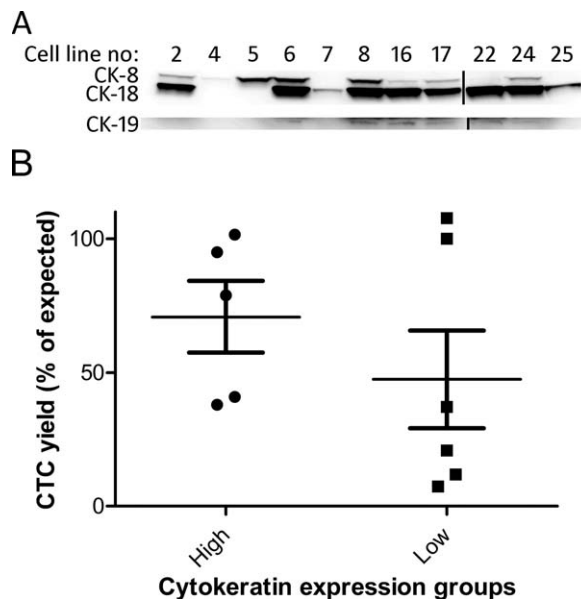

Fig. 4. The expression of cytokeratin (CK) 8, 18, and 19 in a selection of head and neck squamous cell carcinoma cell lines. A. The cell lines were lysed and analyzed by Western blotting (N = 1). B. Group analysis comparing the counting efficiency of high (>mean) and low (<mean) CK-8/18 expressing cell lines. The difference was not statistically significant (P = .33, Mann-Whitney U-test).

which cells that were below the system threshold level were detected by the CellSearch operator. Adding the cells with lower-than-threshold staining to the total cell count resulted in a yield of 65% as compared with 53%. This adjustment did not affect the correlation between high and low EpCAM expressers.

DISCUSSION

In this work, we investigated the effect of varying expression of EpCAM on the recovery of cells when counted with the CellSearch method. We used cell lines, as this enabled us to accurately measure the expression of EpCAM and also to control the actual content of cancer cells in the blood sample to calculate the counting recovery. This would not be possible in patient samples. The expression of EpCAM in cell lines could possibly vary depending on growth conditions, but these conditions were kept constant to avoid such problems.

The fact that non-EpCAM expressing tumor cells are not detected in CellSearch CTC counting is inherent to the method as only EpCAM expressing cells are pulled out in the selection process. Thus, already the fact that not all HNSCC tumors express EpCAM indicates a problem and will result in a proportion of false negative test results. In the tested panel of cell lines, only one out of twelve cell lines did not express detectable levels of EpCAM, indicating that this problem might not be of great importance. This is also supported by other studies showing EpCAM expression in a majority of HNSCC tumors.[29,30]

In the present work, we show that EpCAM expressing cells are counted with different yields depending on the actual level of expression. This variation makes it very difficult to ascertain reliable and clinically useful cut-off levels as well as guidance for follow up. In order to use CTC counts as a prognostic or predictive method it is important that absolute measures of CTCs can be reliably assessed. For this, a stable efficiency in counting is central, but this is obviously a problem for HNSCC. On the other hand, assuming that the expression of EpCAM does not vary considerably over time, Cell-Search counting might be used to measure relative effects on the number of CTCs in consecutive measurements, for example, before, during and after surgery.

Tumor cells have been shown to establish new solid tumors more easily if exposed to wound fluid.[4] In a situation where there are adequate surgical margins after resection, circulating tumor cells from the blood stream, which can survive in peripheral blood for hours, can reseed themselves into the very same environment they came from giving rise to local recurrence despite originally successful surgical result.

Earlier results show that a high level of circulating tumor cells indicates a worse prognosis.[31] A continuous detectable level of CTCs after treatment is also prognostically negative as well as rising levels of CTCs after treatment.

Having access to reliable and consistent CTC detection in HNSCC with their inherent heterogeneity and variability would be beneficial for determining prognosis as well as determining adjuvant therapy and guidance to follow-up. The results in this work indicate that it might be problematic to define reliable cut-off values both as a result of variations in EpCAM and cytokeratin expression. The latter problem might be relieved by using other cytokeratin markers but the failure of EpCAM at lower expression levels to quantitatively capture all CTCs in a blood sample decreases the utility of the CellSearch method in this respect.

The detection of CTCs in blood samples is inherently difficult because of low numbers of tumor cells compared to other blood cells and the often heterogeneous composition of cells in tumors and metastases. A number of different methods have been developed, some relying on surface markers for cell enrichment and others on physical characteristics of the tumor cells. For HNSCC, methods that do not rely on the expression of surface proteins might do better. Different methods for CTC enrichment and detection have been reviewed recently,[32] but also other methods, such as acoustofluidic separation, are being developed.[33] None of these have been clinically validated yet.

CellSearch is an approved method for the detection of circulating tumor cells in cancer in breast, colon, and prostate which is understandable since these tumors in general have more homogenous tumor populations and more stable, though malignant, genomes. For a reliable interpretation in HNSCC it is more important to have a consistent result regardless of the varying expression of EpCAM and other surface markers than to have a high detection rate. This is important both in repeated sampling over time as well as when there are low CTC levels where larger margin of error could give a false impression of a negative result.

There is a possibility that if CTC detection in HNSCC was more accurate and took the variability and polyclonality in HNSCC into account the detection and quantification of CTC would be a better prognostic and predictive tool.

CONCLUSION

In conclusion, we show that variations in EpCAM expression in EpCAM positive HNSCC cells results in varying efficiency in CTC counts by the CellSearch method. This probably limits the usefulness of the method in determining absolute CTC counts for this tumor type. On the other hand, the method might still be used for consecutive measurements in the same patient with EpCAM-expressing tumors in which relative changes are used as out-put. The results points at the importance of developing cell selection processes that do not rely on specific surface markers.

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