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The Challenge of Tumor Heterogeneity – Different Phenotypes of Cancer Stem Cells in a Head and Neck Squamous Cell Carcinoma Xenograft Mouse Model

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Abstract. *Background/Aim: Besides late diagnosis, tumor metastasis and cancer relapse are the main reasons for the poor prognosis of patients with head and neck cancer. Several investigations have shown that tumor is of heterogeneous molecularity consisting of several subpopulations, with a broad range of biological behaviors. The ability and potential of tumor to infiltrate into vessels and into neighbouring organs, as well as the resistance to chemotherapeutical cancer therapy may be caused by cancer stem cells (CSCs). The aim of the present study was to illuminate the role and behaviour of (CD44) and (ALDH1A1) as tumor stem cell markers in a xenograft mouse model of squamous cell carcinoma. Materials and Methods: Five female NMRI-Foxn1nu mice were injected with five million Detroit 562 cells (100 μl). After sacrifice of the mice, tumors were excised. Then ALDH1A1, CD44, (EGFR), CD31 and Ki 67 were detected as molecular markers for tumor stem cells by immunohistopathology and immunofluorescence. Results: The amount of putative CSC marker proteins CD44 and ALDH1A1 vary. ALDH1A1^{high} tumor cells express low levels of CD44 and EGFR. The CD44+^{high} expressers also exhibit expression of high levels of the EGFR. CSCs must be sub-classified depending on their expression of marker proteins. Conclusion: We assume that CSCs can also be sub-classified into migratory and stationary CSCs. ALDH1A1^{high}/CD44^{low}/EGFR^{low} tumor cells may be stationary and quiescent, whereas ALDH1A1^{-}/CD44^{high}/EGFR^{high} expressers have a migratory, invasive nature. It is likely that a regulatory mechanism, as yet unknown, controls this conversion, from quiescent to active cancer stem cells.*

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Diagnosis and treatment of head and neck squamous cell carcinoma (HNSCC) arising from the mucosal epithelium of the upper aerodigestive tract remain a great challenge in modern oncology. HNSCC is the sixth most common type of cancer worldwide, with more than 600,000 new cases appearing worldwide every year. Two-thirds of the patients have advanced cancer with a critical outcome (1). Beside late diagnosis, tumor metastasis and cancer relapse are main reasons for the poor prognosis of head and neck cancer patients with HNSCC. In general the tumor mass is of heterogeneous molecular identity, with several subpopulations exhibiting a broad range of biological behaviors (2). The ability and potential for tumor infiltration into vessels and neighbouring organs, and resistance to cancer chemotherapy may be caused by cancer stem cells (CSCs) (3). Independently of hierarchical or stochastic models of cancer heterogeneity, the term CSC may be used to characterize a small group of cells with a stem cell-like phenotype (4). CSCs are tumor cells that are motile, slow cell cycles with the ability to self-renew and differentiate to reform the whole tumor (5). The expression of several proteins correlates with these characteristics. Indeed the expression pattern and level is not consistent. Even in one cancer entity, such differences in expression are present. In cancer research, a subset of such protein markers is often used to build a profile identifying the CSC population.

CSCs can be found in various hematological and solid tumors, such as of breast, colon, and lung carcinomas as well as in head and neck tumors of squamous carcinoma cell origin (HNSCC) (6). In HNSCC, (CD44) and (ALDH1A1) are putative markers for CSCs (7).

ALDH1A1 is a member of the aldehyde dehydrognase family and is a key protein in alcohol metabolism. ALDH1A1 is expressed in stem cells and CSCs. It plays an important role as an antioxidant and in retinoic acid production. The ALDH family is associated with cellular self-protection, differentiation and cellular expansion (8). CD44 is the hyaloronan receptor. It is found in several different splicing isoforms and interacts with

the extracellular matrix. CD44 is present in the stem cell niche and in the membrane of CSCs. It is essential for cellular adhesion, migration and proliferation (9).

The aim of the present study was to illuminate the role and behaviour of CD44 and ALDH1A1 as tumor stem cell markers in a xenograft mouse model of squamous cell carcinoma.

Materials and Methods

Cell culture. Detroit 562 cells (ATCC, Wesel, Germany) were cultured in a monolayer, using serum-supplemented Eagle's minimum essentiell medium [(PAA, Pasching, Austria), 10% fetal bovine serum (FBS)], 0.5 mM sodium pyruvate, 25 mg gentamycin added) at 37˚C with 5% CO_2.

Cells were harvested with enzyme-free cell dissolution solution (Millipore, Merck, Darmstadt, Germany) to avoid enzymatic protein degradation of surface proteins. Cell solution was washed in 1× phosphate buffered saline (PBS). After centrifugation the supernant was discarded and the cell pellet was frozen at –80˚C. The frozen cell pellet was embedded in Tissue-Tek OCT (Sakura, Alphen aan den Rijn, Netherlands) compound and processed as for frozen tumor tissue.

For injection, cells were detached with Accutase (PAA, Pasching, Austria) and the density of living cells was determined with Cedex XS cell counter (Innovatis Roche Diagnostics, Mannheim, Germany). The cells were diluted in Ringer Lactate at a density of 5×10^6 cells/100 μl. The injection solution was transferred on ice to the animal housing facility (University Science center, Frankfurt/Main, Germany).

Mice, tumor xenografts and treatment procedure. Mice were housed in a pathogen-free facility with a 12 h light-dark cycle and free access to food and water. Five week-old female NMRI-Foxn1nu mice (Harlan, Rossdorf, Germany) were anasthetized with Forane (Baxter, Unterschleißheim, Germany), evaporated with Forena Vapor 19.3 (Draeger, Lübeck, Germany). Five million cells (100 μl) were injected *s.c.* into both flanks of each mouse. Mice were killed 12 days after tumor cell transplantation.

Mouse experiments were permitted by Regierungspräsidium Darmstadt, Hessen F66/08.

Staining procedure. After sacrifice of the animals, tumors were excised. One tumor was directly frozen in liquid nitrogen, the second was fixed in Notoxhisto (Quartett, Berlin, Germany) and embedded in paraffin.

In the staining procedure ALDH1A1 (rabbit, ab-52492, 1/50-1/100; Abcam, Cambridge, UK), CD44 (MU310-UC, mouse, 1/100; Bio Genex, San Remo, USA), EGFR (rat, ab-231, 1/100; Abcam, Cambridge, UK) and CD31 (rat, DIA-310, 1/20; Dianova, Hamburg, Germany), Ki 67 (rabbit, KI68IC01, 1/200; DCS, Hamburg, Germany) primary antibodies were used. EGFR and Ki67 staining was performed exclusively on frozen sections.

The incubation with the primary antibody takes 1 h at room temperature. Afterwards we proceeded with DCS Detection Line system (AD050POL-K, PD000POL-R, PD000RP, DD006RAP; DCS, Hamburg, Germany). Staining was developed with 3.3'-Diaminobenzidine reagent (DAB) reagent (DC137C100; DCS, Hamburg, Germany), Fuchsin Substrate Chromogen System (K0625; Dako, Hamburg, Germany) and HistoGreen (E109; Linaris, Dossenheim, Germany). Photographs were taken with a Zeiss Axioplan2 (AxioCam ICc1 camera; Zeiss, Oberkochen, Germany). ALDH1A1 and CD44 expressors were counted on paraffin sections. Five randomly taken photographs (10x magnification) of each tumor were analyzed in Adobe Photoshop CS5 (Abdobe, Dublin, Ireland).

For fluorescent staining, secondary antibodies of goat anti-rabbit IgG (FITC) (111-095-144), goat anti-mouse IgG TRITC (115-025-146) (both Dianova, Hamburg, Germany) and Santa Cruz (Santa Cruz, Heidelberg, Germany) antibody goat anti-rat-IgG Cy5 were diluted 1/200 in (PBS) containing 5% goat serum, 1% bovine serum albumine (BSA) and 0.1% Tween and incubated for 1 h at room temperature. Additional DAPI staining took 10 min. Slides were mounted with fluorescent mounting media (Dako, Glostrup, Denmark) and photographs were taken with Zeiss M2 Axio Imager Microscope (Zeiss, Oberkochen, Germany), exposure time 1-2 s.

Results

Variable expression of CSC markers ALDH1A1 and CD44. Interestingly, the expression of CD44 and ALDH1A1 differed between *in vitro* and *in vivo* cultivation (Figure 1). The majority of the *in vitro* tumor cells express high levels of CD44. In the *in vitro* cultivation of Detroit 562 cells, the number of ALDH1A1-positive cells was extremely low. We repeated staining of a cell pellet section three times and found only two $ALDH1A1^+$ cells in these samples.

Surprisingly, the situation changed completely in tumor xenografts (Figure 1). The majority of tumor cells expressed high levels of CD44. Moreover the number of $ALDH1A1^+$ cells was greater and the majority co-expressed CD44 (DAB staining). Approximately 12% (±4%) of the tumor cells in xenografts were $CD44^+/ALDH1A1^+$ (results based on enzymatic Fuchsin and DAB turnover). Using immunofluorescent staining improved detection of variations in protein expression and helped to divide tumor cells into low and high expressors. Controversially, the more sensitive immunofluorescent staining of tumor xenografts indicated that many cells with high levels of ALDH1A1 had a reduced amount of CD44 (Figure 3). Certainly some tumor cells with concomitant moderate ALDH1A1 and CD44 staining signal were present. But the observed $ALDH1A1^{high}/CD44^{low}$ phenotype seemed to be very prominent. The $ALDH1A1^{high}/CD44^{low}$ tumor cells were located in groups in the central tumor, as well as in the invasive parts at the tumor borders. In cell nests, the $ALDH1A1^{high}/CD44^{low}$ tumor cell group was surrounded by $ALDH1A1^-/CD44^{high}$ expressers.

The location of $ALDH1A1^{high}/CD44^{low}$ tumor cell group within a cell nest was axial shifted. Tumor cells inside of vessels remarkably were $ALDH1A1^-/CD44^+$ (Figure 1).

Unequal expression of EGFR. We also found differences in the EGFR expression in tumor cells (Figure 2). The majority of *in vitro* cultivated Detroit 562 cells expressed high amounts of EGFR. In xenografts, some groups of cells had reduced EGFR expression. These cells were mostly Ki67-

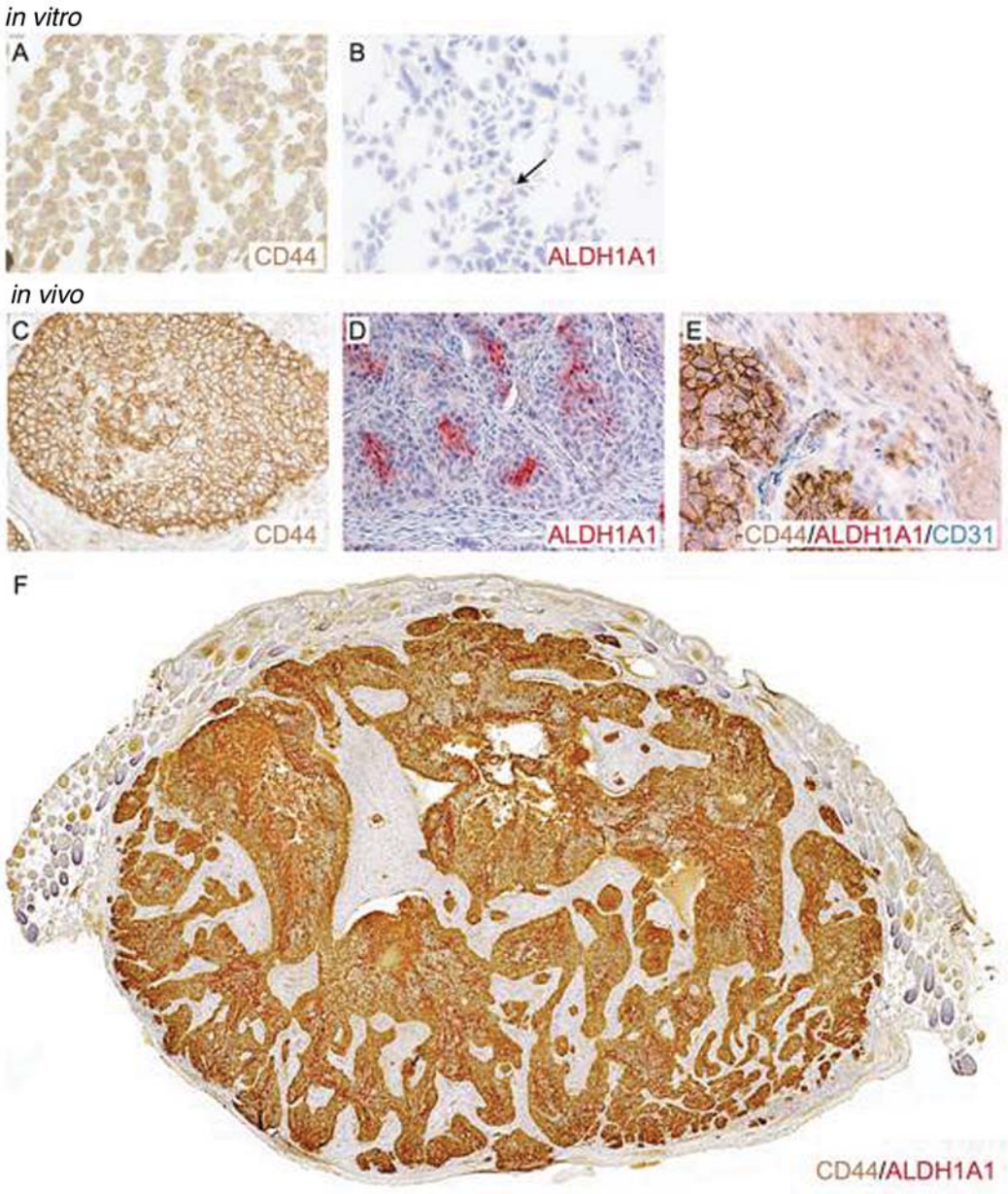

Figure 1. *Expression of Cancer Stem Cell (CSC) marker (CD44) and (ALDH1A1) in cell culture and tumor xenograft. The number of ALDH1A1- and CD44– expressing cells is increased in vivo. Tumor cells in vessels can be seen to express CD44. CD44 expression in vitro ×40 magnification (A), ALDH1A1 staining in vitro ×40 magnification (B), CD44 staining in vitro ×20 magnification (C), ALDH1A1 staining in vivo ×20 magnification (D), CD44 (brown), ALDH1A1 (red) and CD31 (green) staining in vivo ×40 magnification (E), CD44 (brown) and ALDH1A1 (red) co-staining (F).*

negative, which indicates that they were not actively proliferating. Immunofluorescent co-staining for EGFR and ALDH1A1 showed that almost all ALDH1A1^{high} expressers had reduced levels of EGFR (Figure 3).

In contrast, the invasive tumor cells which were found inside vessels, expressed high amounts of EGFR and were ALDH1A1^{-} (Figure 2).

Discussion

Reviewing the present literature with regard to our findings, tumor structure must be divided in several subpopulations. One of the most important units are CSC. Our results give evidence that the group of CSCs can be further subdivided. In HNSCC, the most common CSC markers are CD44 and ALDH1A1 (10).

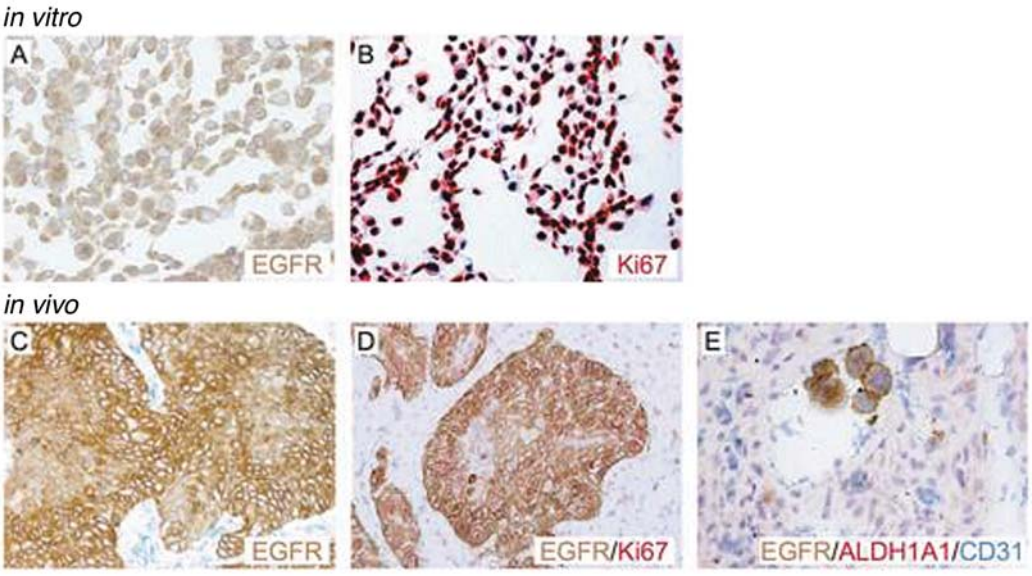

Figure 2. *Expression of EGFR in cell culture and tumor xenograft. Some tumor cells have lower their EGFR expression in vivo. EGFR^{low} tumor cells are Ki 67^{-}. Tumor cells in vessels are EGFR^{high}/ALDH1A1^{-}. EGFR (brown) expression in vitro ×40 magnification (A), Ki 67 staining in vitro (red) ×40 magnification (B), EGFR staining in vivo ×20 magnification (C), EGFR (brown) and Ki 67 (red) co-staining in vivo 20x magnification (D), EGFR (brown), ALDH1A1 (red), CD31 (green) staining 40x magnification (E).*

Using the expression profile of these markers two groups of CSCs could be separated in our approach. Here, we observed that the majority of the tumor xenograft cells were CD44 positive, whereas in cell culture, only a proportion of Detroit 562 cells were CD44^{+}. Pries and colleagues (11) reported that HNSCC cell lines general express CD44 in almost all cells, but did not report on differences in the expression level. Okamoto *et al*. (12) recognized that only 2.1% of conventional cultured (in serum supplemented medium) Gun-1 cells (hypopharyngeal HNSCC cell line) are CD44^{high}. Our results indicate that the ability to express CD44 is important for sufficient cell transplantation into mice and is a sign of invasiveness and tumor survival. Prince and co-workers (13) showed that the CD44^{+} fraction of a HNSCC tumor from a patient, was able to initiate tumor formation in a mouse model. Furthermore, CD44 is a well-accepted marker for CSCs in HNSCC (14).

In our tumor xenografts, the CD44 expressior group overlapped with that of the EGFR expressors. CD44^{high} or EGFR^{high} tumor cells were also present in vessels, indicating the importance of both surface receptors for migration and invasion events, and thus metastasis. Additionally, EGFR^{high} tumor cells were mostly Ki 67- positive. Abhold *et al*. (15) reported that EGFR activation by the EGF ligand induces the expression of several CSC markers, in particular CD44. Wang and colleagues (16) affirmed that the isoforms CD44v3, v6, and v10 mediate the proliferative and migrational activities of CD44^{+} cells in HNSCC. They also claimed that EGFR and CD44 are able to form a complex and act in cooperation. In our approach, only a minority of *in vitro* tumor cells expressed ALDH1A1. Previous research by Chen *et al*. (17) showed that the percentage of ALDH1A1^{+} cells in conventional cell cultures is small. They show that the number of ALDH1A1-positive cells could be increased in spheroid culture. The number of ALDH1A1^{+} cells increased, compared to *in vitro* cell culture, in our tumor xenografts. Because of the cell morphology and location we hypothesize that most ALDH1A1^{+} cells are tumor cells. The slight off-center position of the ALDH1A1^{+} cells in tumor cell nests of variable size, as well as their appearance next to vessel-containing connective tissue, may give evidence that hypoxia and insufficient nutrient supply are not inducers of ALDH1A1 expression. These observations point out that cell interaction could be the reason for the ALDH1A1^{+} phenotype of tumor cells. The majority of ALDH1A1^{high} tumor cells had reduced CD44 and EGFR expressory. Brabletz and colleagues (18) described two forms of CSCs, stationary and migratory. Thus it could be hypothesized that ALDH1A1^{high} expressers are resting, nonmoving CSCs. The EGFR and CD44 surface receptors act in cellular proliferation and migration. Consequently ALDH1A1^{high} tumor cells would be in a generally inactive state or have a retarded cell cycle. These assumed stationary CSCs are also present in the invasive parts of the tumor. It can be supposed that an unknown mechanism converts one phenotype into another. Furthermore it is possible that there is a negative correlation

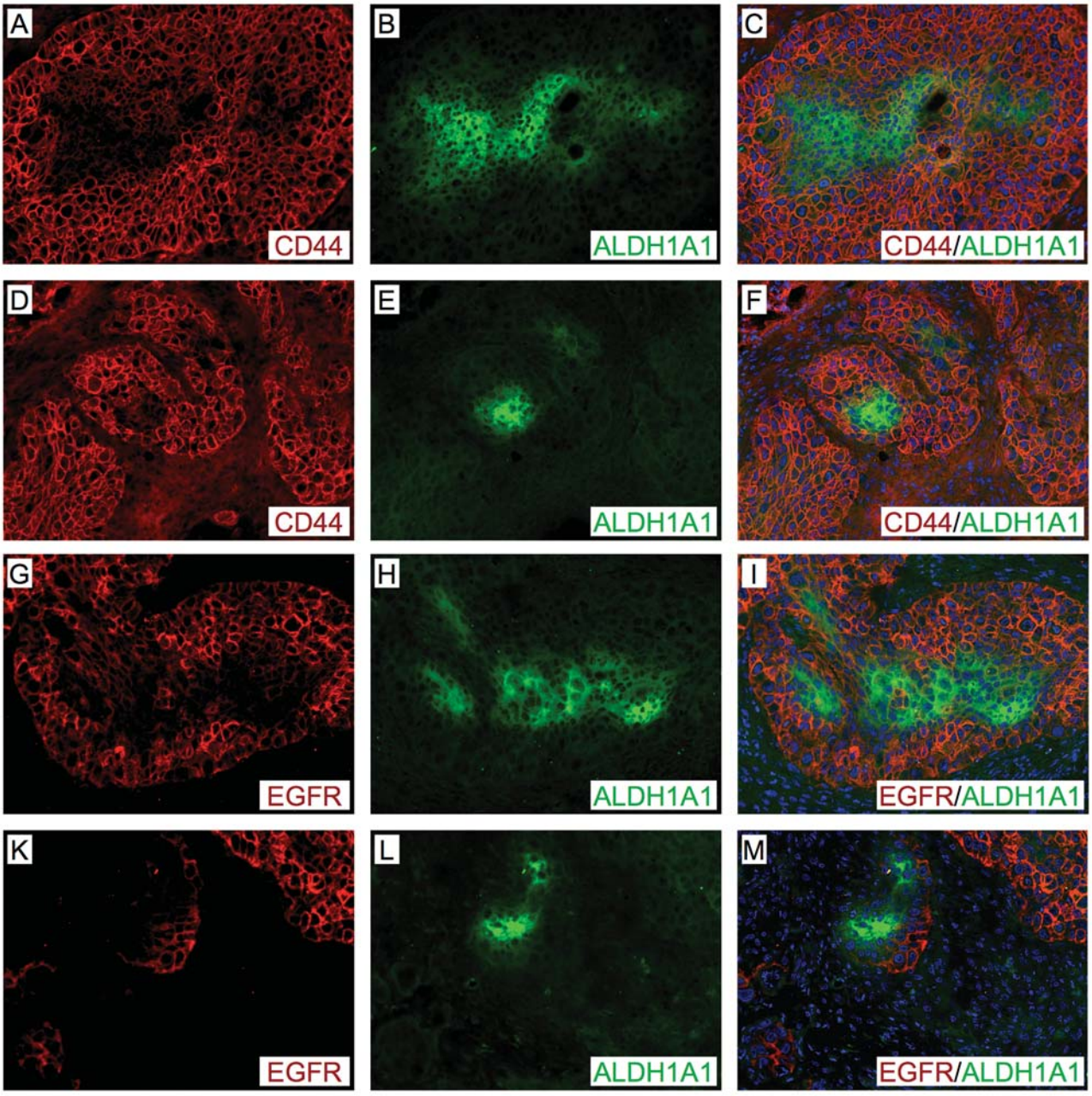

Figure 3. *Immunofluorescent staining CSC markers (ALDH1A1, CD44) and EGFR in cell nests of different extention, ×20 magnification. ALDH1A1^{high} tumor cells are present in large cell nests as well as in smaller clusters in the invasive tumor parts. ALDH1A1^{high} expressers have reduced levels of EGFR and CD44. CD44 (red), ALDH1A1 (green) double staining (A-F), EGFR (red), ALDH1A1 (green) double staining (G-M).*

between ALDH1A1 expression and the state of activation of CSCs. These attributes would make them unaffected by conventional proliferating cell-targeting chemotherapeutic agents (8). Additionally, EGFR-targeting biological agents will also have no effect because of the down-regulation of this receptor. The presence of assumed sleeping cells at the tumor borders, undetectable by conventional histopathology, also makes it difficult to remove them by surgery. This could be one reason for histopathologically negative biopsies within a macroscopic clearly tumorous area. These tumor cells may have the potential to survive cancer therapy and cause relapse. That co-expression of CD44 and ALDH1A1 reveals that the CSCs are well accepted in the literature. Chen and colleagues (19) showed that the direct transplantation of patient tumor

cells that are $CD44^+/ALDH1A1^+/CD24^-$, was more efficient in tumor formation in a mouse model than was $CD44^+/ALDH1A1^-/CD24^+$ or general $ALDH1A1^+$ cell injection.

Conclusion

In our approach, we transplanted HNSCC cell line-derived tumor cells into mice. We found that under these conditions, the expression of putative CSC marker proteins CD44 and ALDH1A1 varied. $ALDH1A1^{high}$ tumor cells express low levels of CD44 and EGFR. The $CD44^{high}$ expressers also had high expression of the EGFR. We assume that CSCs can be sub-classified into migratory and stationary CSCs. $ALDH1A1^{high}/CD44^{low}/EGFR^{low}$ tumor cells may be stationary and quiescent, whereas $ALDH1A1^-/CD44^{high}/EGFR^{high}$ expressers have a migratory, invasive nature. It is likely that an, as yet, unknown regulatory mechanism controls this conversion.

Further experiments will investigate the biological nature of $ALDH1A1^{high}$ tumor cells to determine if they truly are "sleeping" CSCs, as supposed. Our preliminary results also indicate that *in vitro* experiments cannot be always easily translated into *in vivo* settings.

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