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Evaluation of CD44 variants expression in oral, head and neck squamous cell carcinomas using a triple approach and its clinical significance

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Summary

Cancer stem cells possess the qualities of self-renewal, tumorigenesis and the ability to recapitulate a heterogeneous tumor. Our group was the first to isolate head and neck squamous cell carcinomas (HNSCC) stem cells using the cell surface marker CD44. CD44 is a trans-membrane glycoprotein with a multitude of key-functions that regulate cancer cell proliferation and metastasis. The variety of CD44 functions is due to tissue-specific patterns of glycosylation of the extracellular portion, and to the multiple protein isoforms (CD44 variants, CD44v) generated by alternative splicing. This study investigates the expression pattern of CD44 variants in HNSCC. Ten cell lines from most common HNSCC locations and representative of various clinical outcomes were assayed by quantitative real-time PCR, flow cytometry and immunofluorescence comparatively with normal oral keratinocytes. The CD44 v4 and v6 were exclusively abundant in HNSCC while the isoform v1,2 was expressed in normal oral keratinocytes. Of interest, the highest level of CD44v6 expression was detected in advanced metastatic HNSCC suggesting a link between CD44v6 expression and HNSCC metastasis while highest CD44v4 was detected in a stage IV HNSCC refractory to chemotherapy which developed recurrence. Oral-derived HNSCC expressed highest CD44v4 and v6 and levels corresponded with staging, showing also an increasing tendency with recurrence and metastasis. CD44v were detected predominantly in smaller cells (a characteristic that has been associated with stem cell properties) or cells with mesenchymal morphology (a characteristic that has been associated with the migratory and invasive potential of epithelial tumor cells), suggesting that CD44v differential expression in HNSCC may be representative for the morphological changes inherent during tumor progression

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towards a more aggressive potential, and thus contributing to the individual tumor biology. The mechanism of CD44 variants involvement in HNSCC progression and metastasis is under investigation.

Keywords

Head and neck squamous carcinoma (HNSCC); cell lines; CD44 variants; cancer stem cells; clinical outcome; metastasis

Introduction

Head and neck squamous cell carcinomas (HNSCC) are marked by their high aggressiveness and invasiveness (1,2). HNSCC is associated with poor clinical outcome and mortality rates among the highest of all carcinomas (2). As epithelial tumors, HNSCCs contain a heterogeneous population of cancer cells. The cancer stem cell (CSC) hypothesis has attributed this cellular heterogeneity of epithelial tumors to the stem-like properties of a subpopulation of cells within the tumor; this subpopulation through differentiation and uncontrollable division can drive tumorigenesis give rise to the bulk of the tumor (1). Our group has identified CD44 as a cell-surface marker that can enrich a population with stem like properties (1). Cancer cells with high CD44 reactivity, CD44^{high}, typically comprise <10% of the cells in a HNSCC tumor. CD44^{high} cells but not CD44^{low} cancer cells give rise to new tumors *in vivo* and co-stain with the basal cell marker Cytokeratin 5/14. The tumors that arise from sorted CD44^{high} cells reproduce the original tumor heterogeneity and can be serially passaged, thus demonstrating both, an ability to self-renew and an ability to differentiate. Furthermore, the tumorigenic CD44^{high} cells differentially express the *BMI1* gene, a marker of self-renewal and tumorigenesis (1). Large numbers of CD44^{high} cells are found within primary tumors and are required to grow tumors in the mouse model (1, 3–5) suggesting that CD44 variants and its differential expression may provide a better marker for tumor stem cells.

CD44, the major receptor of hyaluronan, is a cell-surface glycoprotein involved in cell-cell interactions, cell migration and adhesion, with multiple isoforms (splice variants) known to be associated with cell transformation and tumor dissemination (5–10). CD44 mediates cellular adhesion, signal transduction and the linking of extracellular matrix with the cytoskeleton. The major source of post-translational modification of CD44, covalent binding of chondroitin sulfate side chains, depends on the pattern of exon splicing (11). These splice isoforms, or CD44 variants display varying ligand binding properties and have been implicated in tumorigenesis and metastatic progression for a variety of tumors (5, 12–14).

CD44 protein is also expressed in soluble form (solCD44), which is detectable in normal circulation (15–20). Circulating levels of solCD44 correlate with metastasis in some tumors (5, 17). It has recently been shown that solCD44 levels in the saliva of HNSCC patients may have potential as a disease marker (21). In this study, we investigate the differential expression profile of the CD44 isoforms in HNSCC, to assess whether a CD44 variant may be a better marker than standard CD44 for HNSCC stem cells and to examine the plausibility of its use as possible diagnostic and prognostic tool.

We have tested the levels of the standard CD44 and of various CD44 variants in a panel of HNSCC cell established by our group (22, 23) (UMSCC 10A, 10B, 11A, 11B, 22A, 22B, 47, 103, 14A, 14B; See table 1 in ref 23) by flow cytometry. We compared RNA expression levels of variant isoforms containing exons 6,7 (V1,2) or 10 (V4) or 12 (V6) in these cells with those of Normal Oral Keratinocytes (NOK) by quantitative real time RT-PCR and verified the presence of the v4 and v6 isoforms with variant specific antibodies by immunohistochemistry and Fluorescence Activated Cell sorting (FACS). Development of these techniques is necessary for studying the biological mechanism of CD44 contribution to tumorigenesis.

This work reveals that while normal oral keratinocytes express the CD44v1,2 variant form of CD44, CD44v4 and CD44v6 are associated with HNSCC tumorigenesis. Of interest, the highest expression of CD44v6 was detected in advanced metastatic HNSCC suggesting a link between CD44v6 expression and HNSCC metastasis, while highest CD44v4 was detected in a stage IV HNSCC refractory to chemotherapy which developed recurrence. Flow cytometry analysis indicates that oral-derived HNSCC expressed highest CD44v4 and v6 levels relative to staging, while also showing an increasing tendency with recurrence and metastasis. While these principles are true for the oral HNSCC, the expression of another variant may account for the high expression of CD44 observed in non-oral HNSCC with the standard antibody observed by FACS.

We show by immunofluorescence that variant forms of CD44 are predominantly expressed in cells with a distinct mesenchymal phenotype among cells cultured from a highly aggressive oral cancer, while an antibody recognizing all CD44 is delineating the plasma membrane in CD44^{low} cells and the filopodia required for normal cell to cell contact. In a cell culture of highly tumorigenic oral cancer, UMSCC-14A, the overall cell phenotype is more mesenchymal and the antibodies against the CD44v4 and CD44v6 recognized highly polarized, dendritic-like cells. Additionally, we observed the presence of CD44 variant forms in smaller cells, a characteristic that has been associated with stem cell properties (14).

Taken together, our study points to the heterogeneity of the CD44+ populations in HNSCC, which may be indicative of a more aggressive potential. This highlights the need for understanding the biological significance of each isoform and suggests that typing tumor biopsies for their CD44 profile may provide important information on their clinical prognostic.

Materials and Methods

Cell lines and cell culture

All cell lines used in this study were established at the University of Michigan (UM) under a protocol approved by the IRB Office in accordance with the University's regulations (22). Collection of cancer specimens from subjects at the UM Hospital, Ann Arbor was approved through the appropriate UM review boards with written informed patient consent. The cell lines (University of Michigan Squamous Cell Carcinomas, UMSCC) used in this study are established by our group (22). Table 1 summarizes their origin, staging, and treatment

subjected to before derivation. All carcinoma cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA), 1% nonessential amino acids, 100× (Invitrogen), and 1% Penicillin-Streptomycin, 100× (Invitrogen). The cell lines tested negative in periodic monitoring for mycoplasma. The cell lines were genotyped to rule out cross-contamination and to confirm their identity and their morphology was regularly examined. Human Normal Oral Keratinocytes (NOK, ScienCell Research Laboratories, Carlsbad, CA) were used as controls and were grown in basal keratinocyte growth media (KGM) supplemented with hydrocortisone, insulin (bovine), human epithelial growth factor and bovine pituitary extract, in accord with the manufacturer's instructions (Lonza, Walkersville, MD).

Real Time RT-PCR (qRT-PCR)

RNA was extracted from the HNSCC cell lines using the PCR RNAqueous kit (Ambion, Austin, TX) in accord with the manufacturer's instructions. Superscript III (Invitrogen, Carlsbad, CA) generated cDNAs were verified for CD44 expression (Platinum PCR, Invitrogen); beta-actin was used as internal standards. qRT-PCR was performed on a 7900H ABI machine using Roche Fast Start™ SYBR Green mix (with ROX) according to the manufacturer's instructions. CD44 and its isoforms values for carcinomas cell lines were normalized against corresponding values of NOK cells. Values were averaged and standard deviations were included to the reported means.

Fluorescence Activated Cell Sorting (FACS)

Sorting experiments were performed at the Flow Cytometry Core Facility at the University of Michigan on a BD Biosciences FACS Vantage Cell Sorter. The single-cell suspension were washed, counted and then re-suspended to a concentration of 10^6 cells per 1 milliliter. Washes and incubation with antibodies were carried out in Hank's Balanced Salt Solution (HBSS) with 2% heat inactivated calf serum (HICS). Numbers of total viable cells were assessed by 4,6-diamidino-2-phenylindole (DAPI, at 0.5 ml per 10^6 cells; BD Pharmingen) exclusion. The following antibodies were used: anti-CD44 (allophycocyanin APC-conjugated mouse anti-human, clone G44-26; BD) and anti-human CD44v4 and v6 antibodies (R&D Systems, Minneapolis, MN) which were detected by APC633 and Alexa Fluor488 anti-mouse antibodies (Invitrogen). All antibodies were used at a final concentration of 2 microgram/milliliter. Gates were set as follows: samples incubated without antibody were used to account for autofluorescence; samples incubated with the isotype control antibodies (allophycocyanin APC-conjugated mouse IgG2b κ , clone 27-35 and FITC mouse IgM, BD) were used to control for nonspecific binding. Viable cells assessed by DAPI exclusion were gated for forward and orthogonal light scatter with filters appropriate for the fluorescent antibody tag. The 10 to 15% of cells with the highest CD44 expression were collected as CD44^{high}, while the 10 to 15% with the lowest or no expression as CD44^{low} cells (3).

Immunofluorescence

Cells were grown in slide chambers (BD Pharmingen) then fixed with 4% formalin for 30min on ice. Image FX™ enhancer (Invitrogen) was used prior to blocking with normal rabbit serum (5µg/ml) and BSA (2% by weight) in PBS. Antibodies were used as above, incubated

in blocking solution, washes in PBS. Images were obtained on a Nikon scope equipped with an Olympus camera and processed on Olympus DSP Image Manager.

Results

CD44+ levels in UMSSC cell lines

Overall, our data indicate that higher CD44 levels detected by FACS correspond with higher levels of tumor aggressiveness. The highest CD44 reactivity was shown for the matched UMSSC-14A&B oral-derived cell lines; -14A was derived from a carcinoma of the floor of the mouth and -14B is subsequent local recurrence of the primary tumor; -14A&B are highly tumorigenic in *in vivo* models (22). The lowest value for CD44 reactivity was detected in UMSSC-10B line; -10A was established from the primary lesion of a larynx carcinoma and -10B from its corresponding lymph-node metastasis; -10A line was about 10× more resistant to conventional treatment compared to the -10B lymph node metastasis cell line (22). In the paired HNSCC UMSSC-10, -11, -14 and -22, the CD44 reactivity was highest in the “B” lines than in their corresponding primary “A” lines. UMSSC-11A originated from a primary larynx tumor and -11B from its corresponding local recurrence; UMSSC-22A was established a primary hypopharynx carcinoma and -22B from its nodal metastasis (22). The relative levels of CD44 positivity in the tested cell lines are illustrated in Fig. 1.

CD44 isoforms expression in HNSCC cell lines (normalized to NOK)

CD44 is expressed in normal epithelial cells and differential expression of splice isoforms (so-called variants, CD44v followed by a number) resulting from alternative exon splicing has been demonstrated in epithelia (25). The structure of the CD44 gene with exons marked and showing variant forms corresponding to the presence of each exon is shown in Fig. 2d. The standard form of CD44 expressed in lymphocytes does not contain exons 6–16, while variant forms are labeled for retaining a specific exon but the final form of spliced transcript and derived protein may vary and is not known. The V1,2 primer set used in the study of RNA levels identifies exons 6 and 7. The V4 identifies the presence of exon 10 and the V6 the presence of exon 12.

The relative levels of CD44v1,2, v4 and v6 in our panel of HNSCC cell lines are illustrated in Fig. 2a–c. Our data indicates that v1,2 isoform is expressed in NOK cells and is elevated in the UMSSC cell lines tested. Its expression however is significantly lower in the UMSSC-14A&B cell lines showing that splicing in these cells is different than in normal oral keratinocytes. The highest value for CD44v1,2 was detected in UMSSC-10B cell line which also exhibited the lowest CD44 reactivity by FACS with the pan-CD44 antibody. NOK levels of CD44 transcripts containing exons 10 (CD44v4) and 12 (CD44v6) were undetectable by quantitative PCR and were assigned the arbitrary value of Ct = 42 for quantification purposes. Cell lines UMSSC-10A and its corresponding lymph node metastasis -10B expressed the highest level of isoforms containing exons 6,7 (CD44v1,2) as well as exon 12 (CD44v6), with -10B expressing 2.5× as much CD44v1,2 (42-fold change over NOK for -10B versus 17-fold change for -10A) and 4.43× more CD44v6 (25709-fold change versus 5792-fold change). UMSSC-11A, an advanced stage (IV) primary carcinoma resistant to the conventional treatment regimens, had the highest expression of CD44v4

while UMSSC-10B, a high stage metastatic carcinoma, expressed the highest CD44v6. UMSSC-14A&B, early stage (I) oral carcinomas cell lines, had the most moderate increases in all isoforms with -14A expressing 40-fold more CD44v4 than NOK. It is conceivable that in this cell line most CD44 is in the standard form or of another isoform that we did not test for (e.g. V7). However, all the CD44 variants tested as well as the overall relative levels of CD44+ were higher in -14A than in its corresponding recurrence -14B.

Overall, real time quantitative RT-PCR was successful in detecting variant forms of CD44 that were not present in normal oral keratinocytes. Our data also indicates that in both oral and non-oral derived HNSCC, the recurrence-forming tumors express both CD44v4 and v6 higher in their primary tumor than in their corresponding recurrences. The levels of expression increased with the tumor stage and were highest in the non-oral derived HNSCC cell lines. In the metastatic tumors, those variants were higher than in their primary counterparts HNSCC.

Relative CD44^{ve} cells recognized by FACS analysis

Next we proceeded to test how well the q RT-PCR data were reflected in FACS analysis using antibodies against all CD44 (pan-CD44) or specifically against its variants. We found CD44v4 levels detectable by FACS in the oral-derived UMSSC-14A and -103 lines but not in the larynx-originated UMSSC-11B cells, while CD44v6 was detectable in all of them (Fig. 3). Levels of variant forms in long term established cell lines were significantly lower than in freshly established ones such as UMSSC-103 cells which were recently isolated from a very aggressive metastatic HNSCC primary oral carcinoma (3). Expression of CD44 as detected by the pan-CD44 antibody is 19 times higher than that detected by the CD44v6 antibody in UMSSC-11B cells (15.2 versus 0.8), 44 times higher (48.1 versus 1.1) in UMSSC-14A cell line and 1.7 times higher in UMSSC-103 cells (32.1 versus 18.3). The CD44v4 isoform was not detectable by FACS in UMSSC-11B cells, and the CD44v1,2 isoform was expressed 29 times less than CD44v1,2 isoform in UMSSC-14A cells and only 2.7 times less than the CD44v1,2 in UMSSC-103 cells. Further analysis will require investigation of the effects of tissue culture on CD44 expression in UMSSC cells over time comparatively with its differences in the corresponding primary tumor fresh biopsies.

Flow cytometry analysis indicated that oral-derived HNSCC expressed highest CD44v4 and CD44v6 and levels corresponded with staging, showing also an increasing tendency with recurrence and metastasis (Fig. 3).

Using our standard protocol, the 10–15% of cells with the highest expression (harboring the cancer stem cell population) were isolated and collected by FACS as CD44^{high} and the 10–15% with the lowest or no expression were usually collected as CD44^{low} cells, as we previously reported (3). The levels of CD44 isoforms in CD44^{high} versus CD44^{low} cells from the oral-derived stage IV metastatic (both distant and in the lymph nodes) UMSSC-103 cell line are illustrated in Fig. 4. qRT-PCR was run to compare isoforms expression in CD44^{high} versus CD44^{low} cells. We found that expression of variants v4 and v6 was enriched in the CD44^{high} compartment by 4 and 2 fold correspondingly, while the v1,2 isoform was expressed equally in CD44^{high} versus CD44^{low} cells.

CD44⁺ reactivity visualized by immunofluorescence in HNSCC

The immunostaining results indicate that the oral-derived HNSCC cell lines exhibited highest CD44 expression as detected by FACS analysis (Figs. 4 and 5). Immunofluorescent microscopy also showed differential pattern of expression for the pan-CD44 and the variants antibodies (Fig. 4 d–f & g–i). While the standard form of CD44 delineates the plasma membrane and cell-cell boundaries, the variants seem to mark cells with a more mesenchymal morphology and a smaller size. All forms of CD44 but mostly CD44v1,2 are seen in higher magnification to participate in filopodia. UMSSC-103 cells showed CD44v1,2 reactivity at the plasma membrane and delineating the filopodia. The filopodia are only detectable if a Triton-X permeabilization step is omitted during the immunocytochemistry. CD44 staining in dividing cells labeled the cellular apparatus involved in cell division, and specifically the centers of organization of microtubules at the poles of dividing cells (centriole). Reactivity by immunocytochemistry did not pick up any variant forms for this cell line, indicating that both FACS and real time RT-PCR have higher sensitivity. Fluorescent immunocytochemistry seems most valuable in providing an insight for the potential biological functions of CD44 as extrapolated from their pattern of cellular distribution. In the oral-derived cell line UMSSC-14A antibodies against the CD44 variant forms gave strong signal for cells with a highly polarized, almost dendritic cell profile and were very rare among the general population of cells consistent with the qRT-PCR data.

Discussion

CD44 is a cell-surface marker that can enrich a population with stem cell-like properties in HNSCCs (1). CD44^{high} cells, but not CD44^{low} cancer cells, give rise to new tumors *in vivo* that reproduce the original tumor heterogeneity and demonstrate both an ability to self-renew and an ability to differentiate (1, 3, 26, 27). It has been suggested that tumors that could be successfully xenografted in mice represent a more aggressive phenotype (27). The importance of CD44 as a tumor-initiating cell marker was also previously shown in *in vitro* studies rather than studying fresh human tissues (28, 29). Since a great number (5×10^3) of CD44^{high} cells is required to give rise to tumors *in vivo*, we have hypothesized that a more stringent stem cell marker may exist in the CD44 variant isoforms that arise from alternative exon splicing and are usually only seen expressed in tumors (12–14). We examined the CD44 expression profile in HNSCC derived cell lines with the goal to investigate the differential expression of the CD44 isoforms and to examine the plausibility of its use as possible diagnostic and prognostic tool. Recently, soluble CD44 levels in the saliva of HNSCC patients were shown to be a potential disease marker (21). Our group has previously reported that a flow cytometric measurement of CD44⁺ cells may provide a prognostic test for patients with HNSCC (1,3,27). We examined whether real time RT-PCR would also make a plausible diagnostic tool by comparing data obtained by real-time RT-PCR for CD44 variants with data obtained by FACS analysis and immunocytochemistry. Our data have confirmed the real time RT-PCR as a highly sensitive method of CD44 variant detection and have shown that CD44 variants v4 and v6 are expressed exclusively in HNSCC but not in normal oral keratinocytes. FACS analysis was also determined to be a highly sensitive method for the detection of the variant forms of CD44 in HNSCC, but a greater amount of starting material is required, making more difficult to perform as a routine prognostic tool.

FACS data verified the over-expression of the CD44 variants and the value of the real time RT-PCR as a test for CD44 variant over-expression in HNSCCs. Immunohistochemistry provided an additional tool for the evaluation of expression of CD44 variants with a valuable insight into the possible biological function of CD44 and its variant forms. Our study indicated CD44v1,2 as native in head and neck epithelia, being expressed in normal oral keratinocytes as well as in the HNSCC cell lines; it delineates the plasma membrane and numerous filopodia protruding from the cell membrane to create cell to cell contact. In the UM-SCC-11B cell line the filopodia appear significantly more elongated and seem to mediate cell contact in cells that are not in close proximity. CD44 variants are found in cells that are small, a property that has been associated with stem cell properties of oral keratinocytes (24) or cells that are highly polarized, therefore of a more mesenchymal phenotype that has been associated with a potential for invasion (30). The presence of CD44 variant forms in smaller cells or cells with mesenchymal morphology is therefore consistent with the hypothesis that CD44 variants might be differentially expressed in HNSCC. A very interesting finding is the high labeling of centriole-like structures as well as very specific "polarized" areas of cells in the process of cell division among the HNSCC cells. This finding suggests an active role for CD44 in cell division the lack of whose proper control lies at the heart of carcinogenesis. As CD44 mediates cellular adhesion, signal transduction and the linking of extracellular matrix with the cytoskeleton (7–10) in a variety of cells, a rather ubiquitous involvement of CD44 to cancer would be suggested by a central role in cell division and such a biological function merits further exploration. The major source of post-translational modification of CD44 depends on the pattern of exon splicing (11) and the ligand binding properties of splice isoforms or CD44 variants may vary, but overall CD44 isoforms might be involved in polarization of the cell through a communication of the extracellular environment, the cell membrane and the cytoskeleton.

Overall, immunofluorescence staining provided an additional tool for the evaluation of expression of CD44 variants and indicated the presence of CD44 variant forms in smaller cells (a characteristic that has been associated with stem cell properties) or cells with mesenchymal morphology (characteristic of invasive potential of epithelial tumor cells), suggesting that CD44 variants differentially expressed in HNSCC may be representative for morphological changes incurred during tumor progression towards a more aggressive phenotype. Our results indicate that CD44 variants are more prevalent in CD44^{high} cells consistent with the notion that variant forms may be better markers for the cancer stem cells. Previous studies have shown conflicting results regarding the significance of CD44 in head and neck tumors (reviewed by 27); the majority of these studies have employed immunohistochemistry as detection method for CD44 and its variants. Our present study has analyzed their expression by a combined approach using three different methods, immunohistochemistry, flow cytometry and real-time PCR comparatively to normal oral epithelia. Therefore, this study provides a new perspective of the CD44 expression profile and significance in HNSCC which supports and completes previous studies [1, 3, 26, 27].

Taken together, the results of our study pointed to the differential expression of CD44 variants in HNSCC and the potential usefulness of variant detection methods such as real time RT-PCR for the diagnosis of HNSCC in tumor biopsies. Variation in the splicing profile of the CD44 variants is great and may be involved in the individual phenotype of

HNSCC tumors in a causative or outcome predictive manner. At this time we do not know the exact sequence of transcripts or proteins expressed in HNSCC cells or the possible variations. Our team aims at understanding the biological mechanism of CD44 involvement in HNSCC tumorigenesis. Further analysis will require studies of the genetic profile of UMSSC HNSCC cell lines and primary tumors. Detailed genetic profiling of CD44 isoforms may be a useful and even necessary step in the future diagnosis of HNSCC; paired with an understanding of the biological mechanism of action of CD44 and of each of its variants, and the controls on the splicing mechanism that produce such variants, could conceivably revolutionize the approach to HNSCC diagnosis and treatment. Emerging recent evidence indicates that targeting the individual CD44 variants may hold great promise for therapeutic strategies in cancer patients [reviewed in 31].

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Figure 1. Relative levels of CD44 cells (recognized by pan CD44 antibody)

A. Relative CD44 percentages in UMSSC cell lines recognized by the pan-CD44 antibody and processed by FACS as described in Methods. The population includes cells with any level of CD44 expression. Fig. 1a shows a graph of percentages of the gated live cell population that exhibited CD44 reactivity with a pan-CD44 antibody, with the threshold for expression defined by staining with an isotype control. Live cells were gated for DAPI exclusion and fluorescence in the FITC-range (M1 gate) as shown in Fig. 1b. Maximum

standard deviation between experimental repeats was 8% of the reported value and error bars in Fig. 1a show the maximum error.

B. Representative gating method for Flow Cytometry Analysis. Live cells were gated for DAPI exclusion and Fluorescence in the FITC-range. Percentages of cells gated in the M1 gate in the range of Alexa 488 absorbance are reported here. Isotype control plot and data is compared to signal obtained with the pan-CD44 antibody. The population includes cells with any level of CD44 expression. Maximum standard deviation between experimental repeats was 8% of the reported value.

Figure 2. CD44 isoform expression in HNSCCC cell lines (normalized to NOK) assessed by quantitative PCR

Real Time RT-PCR was performed as described in Materials and Methods.

A. Relative CD44 expression of isoforms expressing exons 6,7 (CD44v1,2) in matched HNSCC cell lines. Fold change was calculated using beta-actin as the internal standard and comparing CD44v1,2 expression of each carcinoma cell line against corresponding values of NOK cells. The fold difference in expression is reported over each cell line.

B. Relative CD44v4 expression in matched HNSCC cell lines. Fold change was calculated using beta-actin as the internal standard and comparing CD44v4 expression of each

carcinoma cell line against corresponding values of NOK cells. The fold difference in expression is reported over each cell line.

C. Relative CD44v6 expression in matched HNSCC cell lines. Fold change was calculated using beta-actin as the internal standard and comparing CD44v6 expression of each carcinoma cell line against corresponding values of NOK cells. The fold difference in expression is reported over each cell line.

D. Structure of the CD44 gene with exons marked and showing variant forms corresponding to the presence of each exon. The V1,2 primers used in the study of RNA levels identifies exons 6 and 7. The V4 identifies the presence of exon 10 and the V6 the presence of exon 12. Drawing is modified from Marhaba R. et al. (11).

	Events R8	% Gated	% Total
Pan	9308	92.6	45.4
V4	187	9.25	2.5
V6	249	8.1	1.7
Isotype	11	0.1	0.03

Figure 3. Relative CD44^{high} cells recognized by FACS analysis for comparison with cellular immunofluorescence results

A. Relative CD44 percentages recognized by the pan-CD44 antibody and antibodies against the V4 and V6 variants as described in Methods. Live cells were gated for DAPI exclusion and Fluorescence in the FITC-range. CD44^{positive} cells are reported here as percentages of the total cell population. Maximum standard deviation between experimental repeats was 8% of the reported value.

B. Representative gating method for Flow Cytometry. Side Scatter versus FITC logarithmic scale for UMSCC-14A cells in the 488nm range. Isotype control plot and data is compared to signal obtained with the panCD44 antibody.

Figure 4. CD44 isoforms immunofluorescence in HNSCC derived from a very aggressive stage IV metastatic oral carcinoma, UMSSC-103

Upper panel: Immunofluorescence microscopy using primary antibodies against isoforms containing exons 1,2 (A–C) and variants v6 (D–F) and v4 (G–I) showed differential pattern of expression for the CD44_1,2 and the variants. While the former delineates the plasma membrane and cell to cell boundaries, the variants seem to mark cells with a more mesenchymal morphology. All forms of CD44 are seen in higher magnification to participate in filopodia. Variants, particularly V6 are also seen in formations similar to intracellular and extracellular vesicles. Dotted arrows denote CD44^{high}, arrow heads denote

CD44^{high} and small size cells, stars shows vesicles recognized by antibody, angled arrow points to filopodia and regular arrow to polarized phenotype.

Lower panel: Quantitative Real Time RT-PCR of UMSCC-103 tumor cells separated by FACS as CD44^{high} versus CD44^{low}. Following our standard protocol (3) the 10–15% of cells with the highest expression (harboring the cancer stem cell population) are isolated and collected by FACS as CD44^{high} and the 10–15% with the lowest or no expression are usually collected as CD44^{low} cells. For each CD44 isoform tested values shown represent relative levels of expression of CD44^{high} compared to values of CD44^{low}.

Figure 5. CD44 isoforms immunofluorescence in HNSCC

Cells from cell lines UMSSC-11B (A–B) and UMSSC-14A (C–E) were analyzed by immunofluorescence microscopy using a pan-CD44 primary antibody along with antibodies against CD44 variants 4 and 6. Filopodia are seen (marked by angled arrows) and a possible contribution of CD44 in structures involved with polarization and cell division is suggested (arrow heads). Nuclei are stained with DAPI. Relative levels of variants for the UMSSC-14A cell line agree with the Flow Cytometry data. Distinct functions may be potentially assigned to variant isoforms as they seem to characterize cells of more polarized phenotype (regular arrows point to cell).

Table 1
Cell lines and their characteristics

The cell lines used in this study and their complete information: the degree of differentiation as shown by cytology (Grade/Differentiation), the stage of the cancer at the time of cell line derivation (Stage), the head and neck site of the biopsy (Specimen Site), whether the biopsy was of primary, recurrent or metastatic lesion (Type of lesion), what the primary site of occurrence had been (Primary Site) and the treatment received by the patient prior to cell line derivation (Previous Treatment).

Cell line	Stage	Specimen site	Type of lesion	Previous Treatment
UM-SCC10A	III	True vocal cord	Primary	None
UM-SCC10B	III	Lymph node	Metastasis	Surgery
UM-SCC11A	IV	Epiglottis	Primary	None
UM-SCC11B	IV	Supraglottic	Recurrence	Chemotherapy
UM-SCC14A	I	Floor of the mouth	Primary	Surgery
UM-SCC14B	I	Floor of the mouth	Recurrence	Surgery, radiation therapy
UM-SCC22A	III	Hypopharynx	Primary	None
UM-SCC22B	III	Lymph node	Metastasis	None
UM-SCC47	III	Lateral tongue	Primary	None
UM-SCC103	IV	Lateral tongue	Primary	None
UM-SCC6	II	Base of tongue	Primary	None
NOK	-	-	-	-