Published in final edited form as:

Oral Oncol. 2013 April ; 49(4): 306–313. doi:10.1016/j.oraloncology.2012.11.009.

CD44 interacts with EGFR and Promotes Head and Neck Squamous Cell Carcinoma Initiation and Progression

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Abstract

Objectives—CD44 is a promising target for therapy in Head and Neck Squamous Cell carcinoma (HNSCC) and has two defined roles in tumorigenesis: it is a cancer stem cell (CSC) marker and it promotes migration and proliferation through interaction with many signaling molecules. The purpose of this study was to investigate the role of CD44 in HNSCC carcinogenesis.

Materials and Methods—The effects of CD44 in cell proliferation, migration, apoptosis and cisplatin resistance were studied by its overexpression in HNSCC cells. We also evaluated the effect of CD44 on tumor progression by siRNA methodology, immunohistochemistry (IHC) and western blot analysis. CD44 and EGFR colocalization were examined in CAL 27 cells by laser scanning confocal microscopy. The interaction between CD44 and EGFR was analyzed by immunoprecipitation.

Results—Overexpression of CD44 enhances cell proliferation and migration and correlates with increased cisplatin resistance and apoptosis inhibition in SCC25 cells. Downregulation of CD44 in CAL27 cells inhibited constitutive EGFR phosphorylation and significantly reduced tumor growth in nude mice. CD44 and EGFR colocalized in CAL 27 cells. CD44 coimmunoprecipitated with EGFR in CAL 27 cells, indicating that these proteins interact with each other.

Conclusion—CD44 therapy in HNSCC may target the CSC population and alter EGFR signaling.

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Conflict of interest statement

Dr Franzmann is co-inventor on a patent(s) focused on CD44 and has the potential for financial benefit from future commercialization. The remaining authors declare no conflict of interest.

Keywords

CD44; head and neck squamous cell carcinoma; cell proliferation; cancer stem cells; treatment resistance

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a devastating illness affecting 50,000 people in the United States and 600,000 worldwide each year (1, 2). The main risk factors include tobacco and alcohol use and HPV virus infection (3, 4). Most of these patients present with late stage disease when cure rates reach only 30%. Despite aggressive treatments with combinations of surgery, radiation and chemotherapy, little progress has been made towards better outcomes.

Several tumor markers are overexpressed in HNSCC including Epidermal Growth Factor Receptor (EGFR), one of four members of the ErbB family. The ErbB family members forms homo and heterodimers that, when dysregulated, initiate tumorigenic signaling cascades (5). EGFR appears to be the most influential ErbB receptor in HNSCC and signals through the Ras-MAPK, PI3K-PTEN-AKT, and phospholipase C pathways (5). Surprisingly, agents against EGFR result in modest response rates for HNSCC. The mechanisms of resistance are still not clear. Point mutations are reported at low frequencies (5) though a truncated, constitutively active mutant EGFRvIII has been reported in 42% of HNSCC analyzed (5) and may help explain resistance. Other mechanisms of resistance include inability to internalize EGFR and cross-activation of the EGFR intracellular tyrosine kinase or downstream molecular pathways such as Ras-MAPK or PI3-PTEN-AKT (5, 6, 7).

Additional targets are clearly needed for HNSCC treatment. Our work showed that soluble CD44, a breakdown product of CD44, is overexpressed in oral rinses from HNSCC patients and distinguishes HNSCC from controls with 62–79% sensitivity and 75–100% specificity (8–10). CD44 was also recently identified as a tumor initiating marker in HNSCC (11). While cetuximab may increase populations of CD44+ and CD133+ tumor initiating cells (12), resulting in resistance, anti-CD44 therapy would be expected to eliminate those resistant populations. CD44 consists of a family of transmembrane glycoproteins with a common domain and a variable region of alternatively spliced exons (exons 5–14) (13). The common domain includes an extracellular region that interacts with growth factors and hyaluronan (HA), and a cytoplasmic moiety capable of interacting with cytoskeletal components including ezrin, radixin, moesin (ERM) and merlin (14–17). Interactions between CD44, HA, and ErbB family members promote tumorigenesis (18–20) and may represent a pathway to EGFR inhibitor resistance by cross-activation of related pathways.

To evaluate the role of CD44 in HNSCC, we have overexpressed it in a CD44 low cell line, SCC25, that forms a differentiating cyst in nude mice (21) and investigated its effects on tumor growth, migration, apoptosis and sensitivity to cisplatin. Then we downregulated CD44 in an overexpressing cell line, CAL 27 and examined effects on xenograft tumor growth and EGFR signaling. We also showed that EGFR and CD44 colocalize and complex together, further implying a signaling interaction. Based on these studies, targeting CD44 may inhibit tumor initiation and alter EGFR signaling.

Materials and Methods

Cell lines

We obtained SCC25, SCC9, SCC15, SCC4, CAL27 (oral HNSCC) and FaDu (pharynx SCC) from the American Type Culture Collection. UM-SCC9 (tonsil SCC) was a gift from Dr. TE Carey, University of Michigan. SCC25, SCC9, SCC15 and CAL27 were grown in DMEM/F12 medium. FaDu was grown in MEM medium. UM-SCC9 and CAL27 were grown in DMEM medium. All cell line media were supplemented with 10% fetal bovine serum, streptomycin and penicillin.

Patient samples

Following informed consent, we obtained tumor tissue from four HNSCC patients, 2 specimens from patients without cancer and 2 tissues taken from histologically confirmed tumor-free margins adjacent to HNSCC in accordance with a protocol approved by the University of Miami Miller School of Medicine IRB. The tissue samples were kept frozen at -80°C .

Western blot analysis of tissue specimens and HNSCC cell lines

To determine CD44 expression of tissue specimens and HNSCC cell lines, immunoblot analysis was performed on cell lysates from HNSCC tissues and cell lines. Cells were lysed with M-PER mammalian protein extraction reagent (Pierce). Then protein extracts from cell lines and tissue specimens were boiled for 3 minutes under reducing conditions. Samples were run on an 8% SDS-PAGE followed by immunoblotting with anti-human CD44 antibody from eBioscience (San Diego, CA); Actin from Sigma (St. Louis, MO); EGFR; Phospho-EGFR (Tyr 1068) from Cell Signaling (Boston, MA) and ErbB2 from Dako (Carpinteria, CA). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody followed by SuperSignal® West Pico Substrate (Thermo Scientific).

Stable transfection and CD44 Silencing on CAL 27 Cells

The siRNA transfections were done using HCAM-CD44 shRNA plasmid (h) and control shRNA-scramble plasmid (Santa Cruz Biotechnology, CA) according to the manufacturer's instructions on CAL27 cells. HCAM-siRNA(h) is a pool of 3 target-specific 19–25 nt siRNAs designed to knock down CD44 expression. The stably transfected cells were selected with puromycin. Overall, seven stable siRNA CD44 knockdown clones were generated and two of them (3C3 and 4B2) used to further study the role of CD44 in HNSCC.

Stable transfection and overexpression of CD44 standard form on SCC 25 Cells

CD44-STD cDNA was cloned on an Invitrogen (Carlsbad CA) expression vector (pcDNA3.1-MycHisA) that allowed stable transfection in the SCC25 HNSCC cancer cell line. Single clones were selected in the presence of G418.

Proliferation Assay

SCC25 stable transfectants and vector controls were cultured, washed, counted, and plated at a cell concentration of 4 000 per well in quadruplicate wells of 96-well plates and kept in culture for 48 h. Cell proliferation was measured in the same plate using the WST-1 colorimetric assay as described by the manufacturer (Roche Applied Science, Indianapolis IN). WST-1 was added to each well and the cells were kept in culture for 3 h. The absorbance of each well was determined at 450 nm.

Migration Assays

For the migration assays, 8µm filters (Becton Dickinson) were used in 24-well culture plates. After cells were serum starved overnight, 10 000 cells in 300 µl of FBS-free DMEM/F12 media were added to the top chamber. The lower chamber contained either 500 µl of 10% FBS-containing DMEM/F12 media or serum free media (to determine a baseline level of migration and as a negative control). Cells were allowed to migrate for 18 hours at 37°C and 5% CO₂. Following incubation cells were stained with crystal violet and migrated cells were incubated with Cell Stain Solution, extracted and detected on a standard microplate reader (560 nm). All nontransfected controls, empty vector transfectant controls, and CD44 transfectants were seeded in triplicate and each experiment was repeated three times.

Chemotherapy Resistance Assay

SCC25 transfectant cells were cultured, washed, counted and plated at 10 000 cells per well in quadruplicate wells of 96-well plates and incubated in DMEM/F12 at 37°C overnight. Then, cells were incubated with cisplatin (Sigma, St. Louis MO) at increasing concentrations (0, 5, 10, 20, 40, 80 µM) for 6 hours, washed twice with media, and incubated for 3 days. The cisplatin effect on the cells was measured using the cell proliferation, WST-1, colorimetric assay.

Annexin/propidium iodine staining after apoptosis induction

To determine the effect of CD44 expression on apoptosis, SCC25 transfectant cells were treated with 1µM Staurosporine (Cell Signaling) overnight. After the treatment, cells were harvested, counted and 1~200 000 cells placed in each tube. The level of apoptosis was determined using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit (San Diego, CA) per the manufacturer's instructions. The level of staining was determined by FACSscan analyses. The non-treated cells were used as controls.

Xenograft studies of nude mice injected with CD44 transfectants

Animal care and experimental protocols were performed in accordance with procedures and guidelines established by the IACUC at the University of Miami Miller School of Medicine. Male (5–6 weeks, n=6) homozygous Nu/J nude mice (Jackson Laboratories, Bar Harbor, ME) were injected subcutaneously with 4 000 000 cells in 100 µl of a mixture of PBS/Matrigel (1:1). Tumors were measured by slide caliper every 3 to 4 days. Tumor volume was calculated by the formula $axb^2/2$ where 'a' is the length and 'b' is the width of the tumor diameter. Animals were sacrificed via CO₂ asphyxiation after 50 days, tumors were excised and processed for protein and histological analysis as described below.

Immunohistochemistry

The tissue specimens were fixed in 10% Formalin buffer for 24 h at RT and embedded in paraffin and 5 µm thick sections were placed on positively charged glass slides (VWR, West Chester, PA). Immunohistochemical stains were performed using Vectastain ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Anti-CD44 antibody (Vector Laboratories, Burlingame, CA), anti-EGFR, anti-Y1068 (Cell Signaling, Danvers, MA) were applied to tissue sections followed by secondary biotinylated antibody and streptavidin-HRP conjugate complex. After washing in buffer, the chromogen diaminobenzidine was applied followed by a counter stain with Mayer's hematoxylin. Negative controls included substituting the primary antibody with preimmune serum from the same species and omitting the primary antibody.

siRNA knockdown of CD44 in nude mice

Male (5–6 weeks) homozygous Nu/J nude mice (Jackson Laboratories, Bar Harbor, ME) were injected subcutaneously (n=6) with 3 000 000 CAL27, 1B3-scramble, and 3C3 and 4B2 cells. Tumor measurements and processing was performed as previously described.

Immunofluorescence

Immunofluorescence was performed using CAL27 cells and paraffin sections of CAL27 xenograft tumors. CAL27 cells grown to confluence on chamber slides (Fisher Scientific) were fixed with 4% paraformaldehyde. Paraffin sections of CAL27 tumors grown on nude mice were deparaffinized with xylene, rehydrated by using graded ethanols, and subjected to heat-induced antigen retrieval with 10mM of sodium citrate buffer (pH 6.0).

After rinsing, CAL27 cells grown on chamber slides and CAL27 xenograft paraffin sections were blocked for 60 minutes at room temperature, and then incubated with primary antibodies. Primary antibodies (CD44 and EGFR) were diluted in 1% bovine serum albumin/0.3% Triton X-100. Then the sections were rinsed and incubated with the secondary antibody conjugated to the fluorescent dye (Alexa Fluor® 488 or Alexa Fluor® 555 from Cell Signaling). The sections were rinsed and incubated with DRAQ5® Dye (Cell Signaling) and then mounted in Slow Fade™ (Invitrogen). Laser confocal microscopy was performed with an LSM 510 microscope from Zeiss (Carl Zeiss GmbH, Germany). Cell monolayers and tissue sections were analyzed and the images collected using the LAS AF software (Leica Microsystems, Buffalo Growth, IL). Each confocal section was obtained as the average of four frames.

CD44/EGFR Co-Immunoprecipitation

CAL27 cells were lysated as described in protocols previously published (18–21). Cell lysates containing 1 mg of protein were incubated 2 hours at 4°C with agarose-conjugated anti-CD44 antibody (2µg/ml; Santa Cruz). Immunoprecipitates were washed with cold buffer, resuspended in Laemmli buffer, and examined for EGFR by Western blot. Immunodetection was performed using mouse anti-EGFR (1µg/ml; BD Biosciences) followed by horseradish peroxidase-conjugated anti-mouse antibodies (2 000 dilution; Cell Signaling). Blots were developed with an enhanced chemiluminescence kit (SuperSignal West Pico; Pierce) according to manufacturer's instructions.

Statistical Analysis

Statistical analyses of comparisons were performed using the student's t-test and GraphPad Prism 5.

Results

CD44 is overexpressed in majority of HNSCC cell lines and mediates proliferation, migration, cisplatin resistance and apoptosis inhibition

Our prior work shows that breakdown products of CD44 are overexpressed in oral rinses from HNSCC patients compared to controls (8–10, 22). CD44 overexpression in HNSCC tissue is also associated with a worse prognosis (23–25). To better understand the role of CD44 in HNSCC tumorigenesis, we characterized the expression of CD44 in HNSCC cell lines using a monoclonal antibody that recognizes all CD44 isoforms. Western analysis of cell extracts showed CD44 is expressed at different levels in six HNSCC cell lines (Fig. 1A) with varying glycosylation patterns and molecular weights ranging from 85–200 kDa. Very low levels of CD44 expression were detected in SCC25 (oral cavity) and FADU cell lines (pharyngeal). Very robust CD44 expression was detected in SCC9, UM SCC9, and CAL27

(all oral cavity) while SCC15 and SCC4 (oral cavity) showed a medium level of CD44 expression.

Since CD44 was recently identified as a tumor initiating marker in HNSCC (11), we created stable clones overexpressing CD44 standard form (CD44-STD) in a CD44 negative cell line, SCC25 (Fig. 1A). CD44 was expressed in two clones 2C2-CD44 STD and 3A2-CD44 STD but not in empty-vector clones 1B1MycHisA and 1B3MycHisA (Fig. 1B).

Next, we examined the role of CD44 in SCC25 cell proliferation and migration. Cell proliferation was measured in SCC25 cells that had been transfected with CD44-STD expression vector, or empty vector (Fig. 2A). Overexpression of CD44-STD significantly increased cell proliferation ($P<0.02$) while SCC25 cells transfected with empty vector control migrated significantly less ($P<0.001$) (Fig 2B), indicating the CD44 standard form plays a role in HNSCC tumorigenesis.

Our next step was to study the effect of CD44 expression on cisplatin resistance. Tumor cell growth was measured using the cell proliferation WST-1 assay in cells that were stably transfected with CD44. The percentage of absorbance relative to control was plotted as a function of cisplatin concentration. The average 50% inhibitory concentration (IC_{50}) for 1B1MycHisA-empty vector was $\sim 10\mu M$ (Fig. 3A) with the same result obtained for the untransfected SCC25 cell line (not shown). In contrast, the CD44-STD SCC25 stable clones (2C2 and 3A2) IC_{50} was shifted from 10 to $\sim 20\mu M$ ($P<0.03$) suggesting that CD44 receptor is involved in tumor cell cisplatin resistance. When cisplatin is above $40\mu M$, the 3A2 clone behaves like the 1B1 clone probably due to high toxicity levels. For this reason, we decided to use the 3A2 clone in apoptosis analysis and in vivo tumor formation.

Another intrinsic property of cancer cells is the ability to resist apoptosis. To test this, we treated SCC25 cells with the kinase inhibitor, Staurosporine to induce apoptosis. The levels of apoptosis were determined by Annexin-V and propidium iodide staining, followed by FACSscan analyses. As shown in Figure 3B, apoptosis in 3A2-CD44 cells was inhibited nearly 50% compared to control 1B1-MycHisA cells, demonstrating that CD44 expression is responsible for the observed suppression of apoptosis.

Overexpression of CD44 in HNSCC xenografts results in more aggressive tumor morphology

Next, we studied the effect of CD44 expression in vivo. When we grew SCC25 CD44 transfectants in nude mice, we were not able to show a significant change in growth rate. However, the SCC25-CD44 transfectants established more efficiently (5/6 implantations verses 4/6 implantations), with significantly larger tumors measured in the transfected group 14 days after implantation (Fig. 4A). Furthermore, the CD44 transfectant xenografts, unlike the more cyst-like SCC25 control xenografts, had a more aggressive morphology with strong CD44 expression and focal keratinization as shown in Figure 4B. These results corroborate prior findings that CD44 plays a role in HNSCC initiation (11).

Downregulation of CD44 by siRNA inhibits the growth of HNSCC xenografts and decreases EGFR signaling

We next evaluated the effect of CD44 knockdown in HNSCC progression. Using a HNSCC cell line, CAL27, that overexpresses CD44 and EGFR, we developed a panel of stable CD44 siRNA clones and scramble sequence controls. Our siRNA CD44 resulted in over 90% reduction in CD44 (Fig. 5A). Knockdown of CD44 significantly reduced the in vivo tumorigenicity of CAL27 in xenograft assays. The 3C3- and 4B2-siRNA clones showed a reduction in xenograft tumor volume 5–6 weeks after implantation compared to the parental

CAL27 and 1B3-scramble controls (Fig. 5B) ($P<0.05$). Overall, these results demonstrate a pronounced effect of CD44 on in vivo tumor growth.

Several studies suggest that CD44 can promote growth factor signaling by high-affinity receptor tyrosine kinases. CD44 isoforms that have the exon v3 are modified by heparin sulfate, enabling them to bind certain growth factors (26, 32–35). To determine if CD44 contributes to EGFR and ErbB2 signaling in HNSCC cells, we used cell extracts from CAL27, CD44 siRNA clones and scramble sequence control 1B3 in western blot analysis. Figure 6 shows that lowering CD44 expression does not affect total EGFR expression in 3C3 and 4B2 clones (and the rest of siRNA clones – data not shown) but caused a 50% reduction (determined by scanning densitometry) in EGFR phosphorylation (Y1068). Total levels of ErbB2 were dramatically reduced (80–90%). The scramble sequence control cells showed the same level of EGFR and ErbB2 expression as the parental cell line CAL27 (Fig 6). These data indicate that CD44 can mediate EGFR and ErbB2 signaling in HNSCC cells expressing high levels of these growth factor receptors.

Since CD44 and EGFR have been implicated in tumor growth and metastasis (18–20), we analyzed their expression in xenograft tissue samples from CAL27; CD44 siRNA clones and scramble control by IHC. Xenografts containing CD44 knockdown clones not only expressed less CD44, but also showed decrease in total EGFR expression and decreased levels of phosphorylated EGFR (Y1068) (Fig. 7). In addition, CD44 and EGFR are highly expressed in most HNSCC tissues as shown in the Western blot in Figure 8 A and B.

Since CD44 expression modulates the signaling of EGFR on CAL27 cells, we decided to analyze their localization by confocal immunofluorescence. Extensive colocalization between CD44 and EGFR was observed in CAL27 cells and its xenograft (Fig. 9). No signal was detected in cells or tissue sections incubated with each secondary antibodies alone or when cells were incubated with primary antibodies followed by no matching secondary antibodies (data not shown). These data demonstrate coexpression and colocalization of CD44 and EGFR in HNSCC cells both in vitro and in vivo.

To test whether CD44 and EGFR interact with one another in HNSCC cells, we performed coimmunoprecipitation experiments using CD44- and EGFR-specific antibodies. Lysates of CAL27 cells were incubated with a rabbit polyclonal anti-human CD44-specific antibody. Western blotting demonstrated that EGFR and CD44 coimmunoprecipitate in CAL27 cell line (Fig. 10). These data are consistent with the notion that CD44 and EGFR interact in HNSCC cells where the two proteins are overexpressed.

Discussion

This work shows for the first time, using HNSCC xenograft models, that introduction of CD44 into tumor cells induces aggressive tumor morphology and appears to enhance tumor initiation. Furthermore, cells overexpressing CD44 show increased proliferation, migration and resistance to cisplatin and apoptosis. Our findings build on recent work suggesting that CD44 expression is common to a subpopulation of HNSCC cells with cancer stem cell properties (28). Moreover, since CD44 is abundant in most HNSCC, it may be an excellent therapeutic target.

Other work in signal transduction supports a role for CD44 in HNSCC tumorigenesis. Authors have recently shown that CD44 complexes with a RhoA-specific guanine nucleoside exchange factor to induce RhoA signaling, phospholipase C epsilon activity and HNSCC cell migration. This complex also interacts with the epidermal growth factor receptor (EGFR) to stimulate Ras-mediated signaling and HNSCC cell growth (18), an interaction that may be related to chemotherapy resistance (19). We showed, for the first

time, that knockdown of CD44 not only decreased CD44 protein expression, but also decreased total EGFR and phosphorylated EGFR in xenograft models. Interestingly, EGFR and CD44 co-localize on the tumor cell surface and also coimmunoprecipitate together, further indicating an interaction between the two molecules. Most importantly, knockdown of CD44 resulted in decreased tumor growth, indicating that CD44 targeting may indeed be effective.

Trials targeting EGFR have shown less efficacy than anticipated (5). Small molecule inhibitors, such as erlotinib and gefitinib, have also been studied, but show little clinical efficacy in HNSCC trials (29). Cetuximab, while more effective than the small molecule inhibitors, only showed response rates of up to 20% (28, 29). Part of the resistance may be due to lack of target expression in the HNSCC stem cell population (30). Targeting CD44 could overcome this problem.

In addition, interactions between CD44 and other ErbB family members promote tumorigenesis (18–20) and may represent a pathway to EGFR inhibitor resistance by cross-activation of related pathways. For example, our work here shows that in addition to down regulation of EGFR, ErbB2 expression is also decreased. Interaction between ErbB2 and CD44 has been shown in other tumors (26, 27) and could potentially provide a mechanism to bypass EGFR inhibition. CD44 is known to interact with many other molecules that may circumvent EGFR inhibition (14, 15, 31). Furthermore, CD44 can be translocated to the nucleus where it interacts with STAT3 and p300 to induce cyclin D1 expression and cell proliferation (31).

Further work must be done to delineate the role of CD44 in HNSCC tumorigenesis. These findings should be confirmed in tissues derived from human tumors. Studies that investigate the specific signaling components would also help clarify whether CD44 plays a role in cetuximab resistance.

Conclusion

Recent reports suggest that CD44 promotes tumorigenesis (26–28). Our work here confirms that CD44 is important for tumor initiation and, when downregulated by siRNA, decreases tumor growth and modifies EGFR signaling. CD44 is potentially useful as a target for therapy. Further work to clarify how CD44 may bypass the EGFR pathway to mediate cetuximab resistance is underway.

Acknowledgments

This work was supported by the National Cancer Institute (R01 CA118584-02) NCI and Florida Bankhead-Coley Pre-Spore grant (09BR01)

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Figure 1.

Expression of CD44 isoforms in HNSCC cell lines. Whole cell extracts (10 μ g) from different HNSCC cell lines (A) and SCC25 clones transfected with CD44-STD expression vector or MycHisA empty vector (B) were immunoblotted with anti-CD44 antibody or anti- β actin antibody as a loading control. Cell extract from SCC9 was used as a positive control.

Figure 2.

Overexpression of CD44-STD enhances cell proliferation and migration. (A) 1B1MycHisA (empty vector) cells or CD44-STD stable transfectants were cultured for 48 hours. Cell proliferation was analyzed by WST-1 colorimetric assay. (B) Using fetal bovine serum (FBS) as chemoattractant agent, migration assays of SCC25 cells transfected with empty vector (1B1MycHisA) and CD44-STD expression vector (2C2 and 3C3) were performed as described in Materials and Methods. Error bars represent calculated standard error of the means. H=hours.

Figure 3.

Overexpression of CD44 correlates with increased cisplatin resistance and apoptosis inhibition. (A) 1B1MycHisA cells (empty vector) and CD44-STD stable transfectants were cultured overnight (ON) followed by treatment with increasing concentration of cisplatin for 6 hours. The *in vitro* growth of these cells was analyzed by WST-1 colorimetric assay. (B) SCC25 cells transfected with empty vector (1B1MycHisA) and CD44-STD expression vector (3A2) were treated ON with 1 μ M Staurosporine. The levels of apoptosis induced by Staurosporine were determined by Annexin-V and propidium iodide (PI) staining followed by FACSscan analyzes. Percentage of apoptotic cells includes cells stained only for Annexin-

V (early apoptosis) and for both Annexin-V and PI (late apoptosis). Errors bars represent standard error of the means.

Figure 4.

Effect of CD44 expression on tumor growth and morphology. (A) 1B3MycHisA or 3A2-CD44 STD cells were inoculated s.c. at 4×10^6 cells/animal into the flank of nude mice. Tumor volume was calculated as described in the Materials and methods. We used 6 animals per group. Data in the graph are mean $\pm$ SD ($p < 0.04$). (B) Hematoxylin and eosin (H&E) and CD44 staining in representative histological sections of 1B1MycHisA or 3A2-CD44 xenograft. CD44 membrane localization was detected only in 3A4-CD44 xenografts. 200 fold magnification.

Figure 5.

Downregulation of CD44 expression following siRNA transfection inhibits tumor growth *in vivo*. (A) Western blot analysis from the different stable clones that downregulates CD44 expression in CAL 27 cells. Densitometric analysis shows maximum loss of CD44 expression in clone 3C3 (93%) compared to clones 3A3 (76%), 3C4 (42%), 4A4 (85%), 4B2 (59%), 4C1 (40%) and 4B3 (5%). (B) Xenograft studies using nude mice (n=6) show a significant reduction in the tumor volume for two of the CD44-siRNA clones compared to vector control (scramble) or CAL 27 ($p < 0.05$).

Figure 6.

Downregulation of CD44 on CAL 27 cells inhibits phosphorylation of EGFR (Y1068) and ErbB2 expression. Reduction of CD44 expression by siRNA inhibits EGFR-phosphorylation and total ErbB2 expression in CAL 27 cells but does not influence total EGFR expression. Lysates were analyzed by SDS-PAGE and Western blotting with anti-EGFR, anti-phospho-EGFR, and anti-ErbB2, or anti-actin antibodies (for loading control).

Figure 7.

Downregulation of CD44 inhibits EGFR expression and its phosphorylation in vivo. CD44, EGFR and phospho-EGFR immunostainings of representative histological sections of CAL 27, scramble vector control (1A3), and CD44 siRNA (4B2 & 3C3) xenografts confirm the effect of CD44 downregulation on EGFR expression and phosphorylation. 200 fold magnification.

Figure 8.

CD44 and EGFR expression on cancer cell lines, xenograft tumors, human normal and tumor tissues. Western blot analysis of extracts from HNSCC cell lines (2C2-CD44STD, FaDu, CAL 27), xenograft tumors from CAL27 cell line (X-CAL 27-1 and X-CAL 27-3), human tumor samples (SCC-43T, SCC-59T, SCC-65T, SCC69T) and normal human tissues (NT-5 and NT-24* - marginal tissue for SCC59T) using CD44 (A) and EGFR (B) antibodies.

Figure 9.

CD44 and EGFR colocalize in HNSCC CAL 27 cells and xenograft tissue. Double labeling of CAL 27 cells (top four panels) and xenograft tissue (bottom four panels) with mouse monoclonal anti-CD44 and a rabbit-anti-EGFR antibody as indicated. Extensive colocalization (orange/yellow in merged images) of CD44 and EGFR was observed in CAL27 cells and xenograft tissue. For nuclear staining we used blue pseudocolor=DRAQ5 (fluorescent DNA dye, C). 630 fold magnification.

Figure 10.

CD44 associates with EGFR in CAL 27 cells. Whole cell extracts (WCE) from CAL 27 cells were immunoprecipitated (IP) with anti-CD44 antibody followed by immunoblotting with anti-EGFR antibody or reblotting with anti-CD44 antibody as an input control. We used normal rabbit IgG as a control for the immunoprecipitation. Neither CD44 nor EGFR was detected in control immunoprecipitation using isotype-matched control antibody.