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CD44 expression denotes a subpopulation of gastric cancer cells in which

Hedgehog signaling promotes chemotherapy resistance

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STATEMENT OF TRANSLATIONAL RELEVANCE

Gastric cancer is the second leading cause of cancer death worldwide and most often presents in an advanced stage. Chemotherapy is often effective but resistance develops quickly leading to limited survival. Here we show that gastric cancers contain a subset of CD44(+) cells with cancer stem cell properties including chemotherapy resistance, and inhibition of the Hedgehog signaling pathway in these CD44(+) cells can reverse chemotherapy resistance. In a clinical trial of chemotherapy with or without the Hedgehog inhibitor vismodegib for advanced gastric cancers, high CD44 expression in tumors treated with chemotherapy alone was associated with worse outcomes while high CD44 expression in tumors treated with chemotherapy plus vismodegib was associated with improved outcomes. Thus the combination of Hedgehog inhibition and chemotherapy may be an effective therapeutic strategy in a subset of gastric cancers with high CD44 expression.
ABSTRACT

Purpose: Gastric cancers (GC) may harbor a small subset of cells with cancer stem cell (CSC) properties including chemotherapy (CT) resistance. The Hedgehog (HH) pathway is a key developmental pathway that can be subverted by CSCs during tumorigenesis. Here we examine the role of HH signaling in CD44(+) GC cells.

Experimental Design: GC cell lines, tumor xenografts, and patient tumors were examined.

Results: GC cell lines AGS, MKN-45, and NCI-N87 grown as spheroids or sorted for CD44(+) were found to have upregulation of HH pathway proteins. HH inhibition using Smo shRNA or vismodegib (VIS) decreased spheroid formation and colony formation. CD44(+) cells, compared to unselected cells, were also resistant to 5-fluorouracil and cisplatin CT, and this resistance was reversed in vitro and in xenografts with Smo shRNA or VIS. CD44(+) cells also had significantly more migration, invasion, and anchorage-independent growth, and these properties could all be blocked with HH inhibition. Clinical tumor samples from a phase II trial for advanced GC of CT with or without VIS were analyzed for CD44 expression. In the CT alone group, high CD44 expression was associated with decreased survival, while in the CT plus VIS group, high CD44 expression was associated with improved survival.

Conclusions: HH signaling maintains CSC phenotypes and malignant transformation phenotypes in CD44(+) GC cells, and HH inhibition can block CT resistance in CD44(+) cells. GC is a heterogeneous disease, and the strategy of combining CT with HH inhibition may only be effective in the subset with high CD44 levels.
INTRODUCTION

With over 700,000 worldwide deaths each year, gastric cancer is the second leading cause of cancer death (1). Except in the few Asian countries such as Japan and Korea where there is endoscopic screening for gastric cancer, the majority of patients with gastric cancer present with advanced disease. Overall survival for metastatic disease is 3-5 months with best supportive care (2). For patients with advanced or metastatic gastric cancer, the response rate to multi-agent chemotherapy is 50% or greater, but nearly all patients develop chemotherapy resistance, and median survival is extended only to 9-11 months (3).

The cancer stem cell (CSC) theory postulates that cancers harbor a subset of cells that share characteristics of normal stem cells, with a capacity for self-renewal and an ability to differentiate into many cell types (4). Numerous studies have demonstrated that purported CSCs are more resistant to chemotherapy than non-CSCs (5). Methods to identify CSCs include tumor formation in immunodeficient mice, spheroid colony formation in vitro, and expression of certain cell surface markers. For gastric cancer, Takaishi et al. tested in six gastric cancer cell lines seven CSC markers and their association with CSC properties. CD44 was the only CSC marker associated with tumor formation in immunodeficient mice and spheroid colony formation in vitro (6).

The Hedgehog signaling pathway is a key regulator of cell growth and differentiation during development (7). There are three Hedgehog genes in vertebrates, Sonic (Shh), Indian (Ihh), and Desert (Dhh), which all bind the same transmembrane receptor Patched 1 (Ptch1) (8). Ligand binding to Ptch1 releases Ptch1’s inhibitory effect on Smoothened (Smo). Smo then enters primary cilia where it promotes the dissociation of the Suppressor of fused (SUFU)/glioma-associated oncogene homologue (Gli1) complex. This allows nuclear translocation of the Gli family of transcription factors (Gli1, Gli2, and Gli3). Gli1 is a strong constitutive transcriptional activator, while Gli2 and Gli3...
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have both positive and negative transcriptional functions (9). Gli transcription factors activate the expression of genes related to cell development, survival, self-renewal, angiogenesis, epithelial-mesenchymal transition, invasiveness, as well as form a feedback loop that enhances or diminishes the Hedgehog response (10).

The Hedgehog pathway is inactive in most normal adult tissues, but Hedgehog pathway reactivation has been implicated in the pathogenesis of several cancers. Activating mutations in the Hedgehog pathway cause a subset of sporadic and familial basal cell carcinomas and medulloblastomas (11). It is estimated that up to one-quarter of human tumors may depend on Hedgehog signaling for growth (12). Berman et al. demonstrated increased Hedgehog pathway activity in esophageal and stomach cancers, and found suppression of cell growth in vitro and suppression of xenograft tumor growth using the Hedgehog pathway antagonist cyclopamine (13).

Given Hedgehog pathway regulation of embryonic development lies primarily through control of embryonic stem cells, Hedgehog pathway regulation of cancer may lie primarily through control of CSC. In this study, we sought to examine the role of the Hedgehog pathway in maintaining certain gastric CSC phenotypes including chemotherapy resistance. We grew three different gastric cancer cell lines as spheroids and found enrichment of not only the CSC marker CD44 but also Hedgehog pathway proteins and certain self-renewal proteins. Inhibition of Hedgehog signaling using shRNA targeting Smo or pharmacologic Smo inhibition with vismodegib blocked spheroid formation. CD44(+) spheroid cells were highly resistant to 5-fluorouracil or cisplatin chemotherapy, and this chemotherapy resistance was reversed with Hedgehog pathway inhibition. Cells grown as spheroids also had increased migration, invasion, and anchorage-independent growth in soft agar, and all these phenotypes were attenuated following Hedgehog inhibition. Hedgehog inhibition in tumor xenografts acted synergistically with chemotherapy to block tumor growth. Finally, analysis of tumor
samples from a clinical trial of chemotherapy with or without vismodegib revealed that the combination of chemotherapy and vismodegib may have improved survival in patients with high CD44-expressing tumors.

MATERIALS AND METHODS

Cell lines and reagents

AGS and MKN-45 are Lauren diffuse-type gastric adenocarcinoma cell lines, and NCI-N87 (subsequently referred to as N87) is a Lauren intestinal-type gastric adenocarcinoma cell line. These cells were obtained from the America Type Culture Collection (ATCC, Manassas, VA). CFPAC-1 and PANC-1 are pancreatic adenocarcinoma cell lines and were also obtained from ATCC. Cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/ml streptomycin, and L-glutamine 2 mM (“regular media”). Cancer cell lines were actively passaged for less than 6 months from the time that they were received from ATCC, and UKCCCR guidelines were followed (14). Vismodegib was purchased from LC Laboratories (Woburn, MA). 5-fluorouracil was purchased from US Biological. Cisplatin was purchased from Enzo Life Sciences (Farmingdale, NY).

Growth as spheroids

Cells were resuspended in DMEM-F12 containing 20 ng/ml of EGF, bFGF, N-2 (1X) and B27 (1X) (“spheroid formation media”) and plated on Ultra-Low Attachment culture dishes (Corning Life Sciences, Tewksbury, MA). Spheroids were collected after 5–7 days except when noted otherwise. Protein was extracted for analysis, or cells were dissociated with Accutase (Innovative Cell Technologies, San Diego, CA) and used for other experiments (15).
Western blot analysis.

Samples were collected in RIPA buffer (Sigma, St. Louis, MO) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN), and protein concentration was determined by BSA assay (Biorad, Hercules, CA). Western blot analysis was performed using the following antibodies: anti-Shh (SC-9024), Ptch1(SC-9016), and CD24 (SC-11406) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Sox2 (#2748), Oct-4 (#2750), Nanog (#3580), and CD44 (#3578) from Cell Signaling (Danvers, MA); anti-Smo (ab38686) and Gli-1 (ab49314) from Abcam (Cambridge, MA); anti-CD133 (CA1217) from Applications, Inc. (San Diego, CA); and anti-β-actin from Sigma.

Fluorescence activated cell sorting (FACS) and magnets cell sorting (MACS)

For FACS, cells were dissociated using Accutase and resuspended in PBS containing 0.5% BSA. The cells were stained with FITC-conjugated CD44 (BD555478) or isotype control antibody (BD555742) from BD Biosciences on ice for 30 min. Cells were then washed with PBS and analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA) using Cell Quest software.

CD44-positive cells were sorted by a magnetic-activated cell sorting (MACS) system (Miltenyi Biotech, San Diego, CA). After collecting spheroids, cells were washed with phosphate-buffered saline (PBS), dissociated to single cells using Accutase, and stained with CD44-Micro Beads on ice for 30 min. Cells were then passed through a LS magnetic column where CD44-positive cells were retained. The CD44-positive cells were then eluted from the column after removal from the magnet. Quantitative analysis of CD44-positive cells was performed by immunofluorescence using FITC-conjugated CD44 antibody.
shRNA

Silencing of Smo was achieved via lentiviral transduction of human Smo shRNA (SC-40161-V, Santa Cruz Biotechnology). A scramble shRNA control (SC-108080) was also used. Maximal knockdown of Smo occurred 72-96 hours after transduction.

Immunocytochemistry

Spheres were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Following cell fixation, cells were incubated with anti-human CD44 (Cell Signaling, Danvers, MA) in a solution of PBS with 1% BSA and 0.1% Triton X-100 at 4 °C overnight. Staining was visualized using anti-mouse Alexa Flour 488 (A11008, Life Technologies, Grand Island, NY). Nuclei were counterstained using 4', 6-diamidino-2-phenylindole (DAPI, Sigma). Stained cells were visualized with a fluorescence-microscope (16).

Immunohistochemistry and Immunofluorescence

Formalin fixed, paraffin embedded sections were deparaffinized by xylene and rehydrated. Immunohistochemistry was performed with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer’s protocol. For antigen retrieval, the sections were placed in citrate buffer (pH 6.0) and heated in a microwave oven for 10 min. For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.3% H₂O₂ in absolute methanol for 15 min at room temperature. The sections were then incubated overnight at 4 °C with primary antibody and washed with PBS containing 0.05% Trion X-100. Incubation with corresponding secondary antibody and the peroxidase-antiperoxidase (PAP) complex were carried out for 30 min at room
temperature. Immunoreactive site were visualized by 3,3'-DAB. Afterward, the slices were counterstained by hematoxylin. Antibodies used were anti-PCNA (Santa Cruz Biotechnology) and CD44 (eBioscience, San Diego, CA). CD44 stain was predominantly localized to the outer cell membrane. CD44 scores (0-300) were calculated by multiplying the staining intensity (0, 1, 2, or 3) by the staining extent (0-100%).

For detection of apoptosis, TUNEL immunofluorescence was performed. Paraffin-embedded sections were deparaffinized and sections were treated with the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) to detect apoptosis following the manufacturer's instruction. Subsequently, sections were stained with DAPI (0.2 μg/ml) for 3 min. Images were analyzed using Molecular Device (Sunnyvale, CA), MetaMorph 7.8.2 software.

For examination of cell lines for CD44 and Gli1, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated with the appropriate primary antibodies in a solution of PBS with 1% bovine serum albumin and 0.1% Triton X-100 at 4 °C overnight. Antibodies used were as follows: human anti-Gli1 (Santa Cruz). Next day, the cells were stained with FITC-conjugated CD44 (BD555478) and anti-rabbit Alexa Flour 594 (Life Technologies). After 2 hr, nuclei were counterstained using 4, 6-diamidino-2-phenylindole (Sigma). Stained cells were visualized with an inverted confocal microscope (Leica Microsystems, Buffalo Grove, IL). Image processing was performed in Imaris 7.6 of Bitplane (South Windsor, CT).

**Single cell colony formation assay**

Spheroids were dissociated with Accutase and monolayer cells were collected with trypsin. For clonal analysis, cells in spheroid formation media were plated into 96-well plates with a density of single cell per well. After 12 h, individual wells were visually
checked for the presence of a single cell. The clones were grown, and colony formation was monitored at 1, 4, 7 and 10 days. Fifty colonies per group were randomly taken to measure colony size, and the size of colonies was measured under an inverted microscope using Molecular device, MetaMorph 7.8.2 software.

Cancer cell proliferation, migration, and invasion assays

Spheroids were dissociated with Accutase and monolayer cells were collected with trypsin. To assay for proliferation, $1 \times 10^4$ cells were plated onto 96-well flat bottom plates and maintained in regular media overnight. A colorimetric MTT assay was used to assess cell number by optical density after 3 days as previously described (17). Data reflect the mean of six samples.

To assay for migration and invasion, cells ($2 \times 10^4$ cells/well) were suspended in 0.2 ml of serum free DMEM for invasion and motility assays. For the invasion assay, the cells were loaded in the upper well of the Transwell chamber (8-mm pore size; Corning, Corning, NY) that was precoated with 10 mg/ml growth factor reduced (BD Matrigel™ matrix – BD Biosciences) on an upper side of the chamber with the lower well filled with 0.8 ml of DMEM with serum. After incubation for 48 h at 37 °C, non-invaded cells on the upper surface of the filter were removed with a cotton swab, and migrated cells on the lower surface of the filter were fixed and stained with a Diff-Quick kit (Fisher Scientific) and photographed at 20x magnification. Invasiveness was determined by counting cells in five microscopic fields per well, and the extent of invasion was expressed as an average number of cells per microscopic field. Cells were imaged with by phase-contrast microscopy. For migration studies, we used invasion chambers with control inserts that contained the same type of membrane but without the Matrigel coating (one chamber per well of a 24-well plate). $2 \times 10^4$ cells in 0.2 ml of serum-free DMEM were added to the apical side of each insert, and 0.8 ml of DMEM with serum was added to the basal
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side of each insert. The inserts were processed as described above for the invasion assay.

**Soft agar and colony formation assays**

Spheroids were dissociated with Accutase and monolayer cells were collected with trypsin. To examine anchorage-independent growth, a cell suspension (2x10^3 cells per ml) was suspended in 0.4% agarose in regular media and seeded in triplicate on 60-mm dishes pre-coated with 0.8% agar in regular media, and incubated at 37°C, 5% CO₂. After 12-30 days, colonies were photographed and counted in four randomly chosen fields and expressed as means of triplicates, representative of two independent experiments.

To assay of colony forming ability, 200-500 cells were plated in regular media onto 60 mm culture dishes and incubated for 7–14 days. Colonies were stained with 0.5 % crystal violet in 6 % glutaraldehyde solution. The number of colonies consisting of 100 or more cells was scored. Data reflect the mean of nine samples.

**Mouse studies**

All mouse protocols were approved by Institutional Animal Care and Use Committee. To generate subcutaneous flank tumor, 5 x 10^6 MKN-45 cells were resuspended in 100 μl of Hank’s balanced salt solution (HBSS) and injected subcutaneously into the right flank of athymic, nude, 6-8 week old male BALB/c nu/nu mice following isoflurane anesthesia. Mice were assigned into treatment groups (6 mice per group) when tumors reached 50 mm³ in volume, designated as day 0. Cisplatin 2 mg/kg or carrier (PBS) was injected i.p. 1 time a week. Vismodegib 100 mg/kg or carrier (PBS) was injected i.p. every day. Tumors were measured three times per week for two weeks, and tumor volume (TV) was calculated by using the following formula: TV =
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length x (width)^2 x 0.52. After mice were sacrificed, tumors were excised and cut into thirds. Portions of each tumor was fixed in 10% buffered formalin for 24 hr, embedded in paraffin, and processed into 5 μm sections.

Statistics

Data represented as mean ± standard deviation (SD). Levels of CD44 between patients were compared by the independent t-test or one-way analysis of variance (ANOVA). Cut-off values for CD44 were determined by analyzing Receiver Operating Characteristics Curves. Overall survival curves were plotted by the Kaplan-Meier method and compared by using log-rank test. A p value less than 0.05 was considered statistically significant. Analyses were performed using IBM SPSS software for Windows version 21 (IBM, Armonk, NY).

RESULTS

Takaishi et al. tested gastric cancer cell lines AGS, MKN-45, and N87 for CSC markers, and only CD44 expression was associated with tumor formation in immunodeficient mice and spheroid colony formation in vitro (6). We first examined expression of CD44 and Hedgehog pathway proteins in these three gastric cancer cell lines and two pancreatic cancer cell lines, CFPAC-1 and PANC1, grown in monolayer conditions or in spheroid formation conditions (Fig. 1A). We examined expression of CD44 and Gli1 in the gastric cancer cell lines by immunofluorescence after placement in spheroid colony formation conditions. CD44 and Gli1 expression was seen at low levels after 1 hr, and expression increased progressively until 72-96 hrs (Fig 1B). By Western blot analysis, levels of CD44 increased in all cell lines when cells were grown as spheroids compared to when grown as monolayers (Suppl. Fig. S1A), and there were varied increases in other
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putative gastric CSC markers such as CD24 and CD133 (Suppl. Fig. S1B). FACS analysis for CD44(+) cells confirmed upregulation of CD44 over time as cells were grown in spheroid formation conditions, with near maximal CD44 levels occurring by 3-5 days in all three gastric cancer cell lines (Suppl. Fig. 1C). The percentage of CD44(+) cells after 22 days in spheroid forming media varied greatly between cell lines from 16.1% in N87 cells to 60.6% in MKN-45 cells. In addition to increased CD44 levels in spheroid cells compared to monolayer cells, Western blot levels of the Hedgehog pathway proteins Shh, Ptch1, Smo, and Gli1 were all higher in spheroid cells compared to monolayer cells (Fig. 1C). We examined expression of these proteins 1-72 hrs after placement into spheroid formation conditions. Shh, Ptch1, Smo, and Gli1 are detected at low levels after 1 hr and levels increased up to 72 hrs (Suppl. Fig. S1D). We also examined levels of CD44 and Gli1 expression following disaggregation of spheroids with Accutase and placement of these cells back into monolayer conditions, and levels of CD44 and Gli1 remained elevated over that of monolayer cells for at least 72 hrs (Suppl Fig. S1E). Levels of the self-renewal proteins Sox2 and Nanog increased in gastric cancer cells when grown as spheroids, but levels of another self-renewal protein Oct -4 remained stable (Fig. 1D). Thus growth of gastric cancer cells as spheroids is associated with sustained increases in levels CD44, Hedgehog pathway proteins, and some self-renewal proteins.

We next examined the effects of Hedgehog pathway inhibition on CD44 expression and spheroid formation. The Hedgehog pathway was inhibited either genetically using Smo shRNA or pharmacologically using the drug vismodegib. Effective knockdown of Smo by shRNA in all three gastric cancer cell lines was confirmed by Western blot analysis (Fig. 2A). Smo shRNA treatment of gastric cancer cells reduced expression of CD44 as measured by immunocytochemistry and also reduced spheroid formation from 20-30 colonies per visual field to 6-10 colonies (Fig. 2B). Vismodegib
treatment also reduced CD44 expression and reduced the number of spheroid colonies from 25-35 to 10-15 per visual field (Fig. 2B). Spheroid cells were next dissociated and grown in a single cells assay under spheroid formation conditions. Hedgehog pathway inhibition reduced the diameter of colonies in this single cell assay after 10 days by 70.3-78.4% for Smo shRNA and 66.9-70.8% for vismodegib (Fig. 2C and Suppl. Fig. S2). Thus Hedgehog pathway blockade inhibits CSC phenotypes such as spheroid formation and colony formation from single cells as well as CD44 expression.

Numerous studies have demonstrated that purported CSCs are more resistant to chemotherapy. We thus separated CD44(+) and CD44(-) cells from AGS, MKN-45, and N87 gastric cancer cell lines using a magnetic bead column (Fig. 3A). Similar to the comparison of spheroid cells versus monolayer cells, CD44(+) cells showed upregulation of Hedgehog pathway proteins Shh, Ptch1, Smo, and Gli1 compared to CD44(-) cells (Fig. 3B). We then examined the sensitivity of these sorted cells to two commonly used chemotherapies for gastric cancer, 5-fluorouracil and cisplatin. For CD44(-) cells, cell viability was reduced by 44-55% when exposed to 5-fluorouracil at 5 μM (Fig. 3C). 5-fluorouracil decreased cell viability in CD44(+) cells by only 13-20%. CD44(-) cells were also sensitive to cisplatin at 5 μM, with reduction in cell viability by 47-54%, while CD44(+) cells were relatively resistant to cisplatin, which decreased cell viability by only 11-22%. The proliferation of CD44(-) cells and CD44(+) from all three gastric cancer cell lines following Smo inhibition with vismodegib was only mildly inhibited with 89-90% and 78-79% viability, respectively (Fig. 3C). The combination of vismodegib and chemotherapy had little or no additive effect on CD44(-) cells. However, there was a highly synergistic effect when vismodegib and chemotherapy were combined on CD44(+) cells, with decreases in cell viability ranging from 80-87%. Similar results were obtained when spheroid cells and monolayer cells were used (Suppl. Fig. 3A) and when
Smo shRNA was used rather than vismodegib (Fig. 3D). We also found a highly synergistic effect of chemotherapy and vismodegib when we examined spheroid cells in a colony formation assay rather than in a proliferation assay (Suppl. Fig. S3B). Thus CD44(+) gastric cancer cells are resistant to chemotherapy, and this resistance can be overcome with Hedgehog inhibition.

We next examined the effects of Hedgehog inhibition on malignant transformation properties including migration, invasion, colony formation, and anchorage-independent growth in gastric cancer monolayer cells and spheroid cells. The three gastric cancer cell lines demonstrated 5.3-6.8 fold more migration (Fig. 4A) and 6.5-13.6 fold more invasion (Suppl. Fig. S4A, S4B) for spheroid cells compared to monolayer cells. Smo inhibition using shRNA or vismodegib reduced the migration of spheroid cells by 50.2-65.6% and reduced the invasion of spheroid cells by 57.4-66.3%. We also measured colony formation ability for monolayer and spheroid cells and found spheroid cells formed 3.8-4.6 fold more colonies than monolayer cells (Fig. 4B). As seen with migration and invasion, Smo shRNA and vismodegib reduced colony formation of spheroid cells by 68.6-72.3% and 38.7-76.5%, respectively. Gastric cancer cells grown as spheroids also demonstrated 4.1-5.5 fold more colonies when grown in soft agar compared to monolayer cells, and the ability of spheroid cells to grow in these conditions was dramatically inhibited with Smo shRNA or vismodegib (Fig. 4C, Suppl. Fig. S4C). Thus gastric cancer spheroid cells compared to monolayer cells have increased migration, invasion, colony formation, and anchorage-independent growth and these properties are dependent on Hedgehog signaling.

The effects of Hedgehog pathway inhibition and cisplatin chemotherapy were examined on gastric cancer xenografts in mice. MKN-45 cells were stably transduced with control shRNA or Smo shRNA, and growth rates of these stable lines in vitro were similar (Fig. 5A). These cell lines were then grown as xenografts in athymic nude mice.
After tumors reached 100-150 mm$^3$ in size, mice were randomized to treatment with cisplatin or PBS. Control tumors treated with PBS grew to over 800 mm$^3$ in just 17 days following randomization. Tumors transduced with Smo shRNA or treated with cisplatin grew to 69% and 56% of control tumor size, respectively (Fig. 5A). The combination of Smo shRNA and cisplatin dramatically inhibited tumor growth, with tumors growing on average from 131 mm$^3$ to 237 mm$^3$ (only 28% of control tumor size) during the treatment period. After 17 days, xenografts were harvested and analyzed. The combination of Smo shRNA and cisplatin did not dramatically reduce proliferation as measured by proliferating cell nuclear antigen (PCNA) immunohistochemistry (Fig. 5B) but did cause synergistic increases in overall apoptosis over cisplatin alone or Smo shRNA alone (25.7 vs. 13.9 and 7.5 apoptotic cells per field) (Fig. 5C). Tumors treated with Smo shRNA and cisplatin also demonstrated a 90.3% reduction in expression of CD44 as measured by immunohistochemistry compared to controls (Fig. 5D).

We performed a similar xenograft study with vismodegib rather than Smo shRNA and had similar results. Cisplatin or vismodegib decreased tumor growth by 52% or 35%, respectively, and the combination of cisplatin and vismodegib decreased tumor growth by 74% (Suppl. Fig. S5A). As seen in the prior xenograft study, tumor cell proliferation was only mildly reduced (Suppl. Fig. 5B), but tumor cell apoptosis increased from 4.7-10.9 cells per field with single agent therapy to 20.9 cells per field with combined therapy (Suppl. Fig. S5C) and CD44 positive cells were reduced to only 23% of control tumors (Suppl. Fig. S5D).

In a randomized phase II study of chemotherapy with or without vismodegib, 124 patients with advanced gastric or gastroesophageal junction adenocarcinoma were randomized to receive 5-fluorouracil, oxaliplatin, leucovorin (FOLFOX) chemotherapy with placebo or with vismodegib (Fig. 6A). There was no difference in response rate, progression-free survival, or overall survival with the addition of vismodegib to
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chemotherapy. We hypothesized that the lack of benefit of vismodegib in this clinical trial was because only the subset of gastric and gastroesophageal junction cancers with high levels of CD44-expressing cells would benefit from vismodegib. We thus performed CD44 immunohistochemistry on 97 available tumor samples from this study and scored the CD44 levels (see Methods). Examples of tumors with low, intermediate, and high CD44 scores are shown in Fig. 6B. The gender and tumor characteristics, radiologic response, follow-up, and survival of these patients are listed in Supplemental Table 1. The median CD44 score was 15.0 (standard deviation 63.6) and the range was 0-270. Two patients in the chemotherapy plus vismodegib group had a complete response, and these two patients had a median CD44 scores of 187.5 compared to 26.4, 31.5, and 42.0 for those with a partial response, stable disease, and progressive disease, respectively (p=0.001). In the chemotherapy only group, patients with no progression of disease had significantly lower CD44 scores than patients with progression of disease, and patients who were alive at the end of the study period had significantly lower CD44 scores than those who had died by the end of the study period (Table 1). Conversely in the chemotherapy plus vismodegib group, patients with no progression of disease tended to have higher CD44 scores, and patients who were alive at the end of the study period had significantly higher CD44 scores. Separate Kaplan-Meier curves were generated for actuarial overall survival of patients receiving chemotherapy alone and chemotherapy with vismodegib. In the chemotherapy only group, estimated overall survival was not significantly different in those with high versus low CD44 scores (Fig. 6C). In the vismodegib group, estimated overall survival of patients with a CD44 count in the top quartile tended to be better than the remaining patients. We performed a univariate analysis of patients in the chemotherapy plus Vismodegib group of patient and tumor characteristics (including tumor location and Lauren type) in relation to overall survival. There were non-statistically significant trends toward improved overall survival
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with high CD44 expression, female patients, diffuse histology, and performance status. Thus Hedgehog inhibition may improve the efficacy of chemotherapy in the subset of advanced gastric cancer patients with high CD44 expression.

DISCUSSION

This study is the first to demonstrate a vital role of the Hedgehog signaling pathway in a subset of gastric cancer cells with CD44 expression in the maintenance of chemotherapy resistance. We grew three different gastric cancer cell lines as spheroids and found enrichment of the gastric CSC marker CD44 as well as increased levels of Hedgehog pathway proteins and certain self-renewal proteins. Inhibition of Hedgehog signaling using shRNA targeting Smo or pharmacologic Smo inhibition blocked spheroid formation. CD44(+) spheroid cells were highly resistant *in vitro* to 5-fluorouracil or cisplatin chemotherapy, and this chemotherapy resistance was reversed with Hedgehog pathway inhibition. Cells grown as spheroids had increased migration, invasion, colony formation, and anchorage independent growth in soft agar, and all these phenotypes were attenuated following Hedgehog pathway inhibition. Hedgehog inhibition in tumor xenografts acted synergistically with chemotherapy to block tumor growth, and histological examination of treated tumors found synergistic increases in tumor cell apoptosis and depletion of CD44(+) cells. Finally, examination of tumor specimens from a prospective, randomized trial of chemotherapy with or without vismodegib in advanced gastric cancer patients revealed that only the subset of these patients with high CD44-expressing tumors may have benefited from the addition of vismodegib to chemotherapy.

The existence of CSCs remains a topic of intense debate. The American Association of Cancer Research Workshop on CSCs defined CSCs as “cells within a tumor that possess the capacity for self-renewal and that can cause the heterogeneous...
lineages of cancer cells that constitute the tumor” (18). The gold standard for identifying CSCs is tumor formation in immunodeficient mice and the best in vitro selection method is growth of cells in serum-free spheroid forming media. Opponents of the CSC theory have argued that xenotransplantation assays may merely select for cells able to grow in a foreign environment (19) or merely depend on the level of immune-compromise of the host (20). More recent studies in genetically engineered mouse models of tumors combined with fluorescent markers to genetically trace CSCs have been performed. Chen et al. demonstrated that a quiescent subset of endogenous glioma cells was responsible for tumor regrowth after chemotherapy (21). Schepers et al. found that for intestinal adenomas, the crypt stem cell marker Lgr5 marks a subpopulation of 5-10% of adenoma cells that can generate additional Lgr5(+) cells as well as all other adenoma cell types (22). There are certainly subsets of cancer cells within heterogeneous solid tumors with varied properties. In this study, we demonstrate one such subpopulation of CD44(+) cells which have the CSC phenotypes of spheroid formation, formation of colonies from single cells, and chemotherapy resistance. However, given the high percentage of CD44(+) cells at baseline in some gastric cancer cell lines and in some human gastric cancers, only a subset of CD44(+) could actually be CSCs.

There is now increasing evidence that Hedgehog signaling is an essential pathway in both hematologic (23) and solid malignancies (24). While increased Hedgehog pathway activation has been demonstrated in gastric cancers (13), there is little prior data on the exact role of Hedgehog signaling in gastric CSCs. Helicobacter pylori infection, a well-known risk factor for gastric cancer, can upregulate SHH in gastric parietal cells and IHH in gastric pit cells (25). Song et al. found that the Hedgehog pathway inhibitor cyclopamine could block spheroid formation in one gastric cancer cell line, HGC-27 (26). In this study, inhibition of Hedgehog signaling in the CD44(+) subpopulation of three gastric cancer cell lines dramatically reduced formation of
spheroids and blocked other CSC phenotypes including single cell colony formation and chemotherapy resistance, thus establishing the vital role of Hedgehog signaling in CD44(+) gastric cancer cells.

CD44 is a transmembrane glycoprotein and the principal cell surface receptor for hyaluronic acid, a major component of extracellular matrix (27). CD44 plays an important role in communication of cell-matrix interactions along with a role in cell motility, matrix degradation, proliferation, and survival. The major form of CD44 on epithelial cells is CD44s (standard) but numerous isoforms of CD44, called CD44v (variant). Several studies have shown an association between CD44v6 and gastric cancer lymph node metastasis, and prognosis (28, 29). In this study, we used CD44 antibodies that recognized all CD44 isoforms and an analysis of specific CD44 isoforms was not performed. CD44 expression denoted a subset of gastric cancer cells that with CSC and malignant transformation properties which could be blocked with Hedgehog pathway inhibition.

There are at least two dozen ongoing or completed phase I and phase II trials using various Hedgehog pathway inhibitors (e.g. vismodegib, BMS-833293/XL139, IPI-926, LDE225, PF-04449913, LEQ 506, and TAK-441) either alone or in combination with chemotherapy for a variety of solid tumors (30). In 63 patients with locally advanced basal cell carcinoma, vismodegib led to a partial response in 22% of patients and a complete response in 21% of patients (31). One lesson learned in our study is that for any given cancer, the Hedgehog pathway may promote chemotherapy resistance in only a subset of patients and that a biomarker may be needed to identify this subset. The clinical trial described in this study of chemotherapy with or without vismodegib for patients with advanced gastric or gastroesophageal cancer was a negative study in that vismodegib did not increase progression-free or overall survival for the group as a whole. However, the minority of patients with tumor having high
Hedgehog signaling in CD44(+) gastric cancer cells   Yoon C et al. -- 21

CD44 expression appeared to benefit from the addition of vismodegib. This association between high CD44 expression and response to vismodegib needs to be confirmed in another prospective clinical trial.

Cytotoxic chemotherapy is a rather blunt instrument in the treatment of metastatic gastric cancer, and the specific pathways that drive the initiation, progression, and metastases of gastric cancers continue to be better delineated as targets. Deng et al. performed a comprehensive survey of genomic alterations in gastric cancer and found the existence of five distinct subgroups defined by signature genomic alterations in fibroblast growth factor receptor 2 (FGFR2), V-Ki-ras Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and c-MET (32). In addition to these five pathways for gastric cancer tumor growth, the vascular endothelial growth factor A (VEGF-A) pathway plays an important role in driving tumor angiogenesis in gastric cancers (33). It remains unclear whether gastric CSCs may also be resistant to targeted therapies. A small percentage of gastric adenocarcinomas overexpress human epidermal growth receptor 2 (HER-2), and the addition of trastuzumab to chemotherapy prolonged survival in these patients from 11 to 14 months in a randomized trial (34). However, combining cytotoxic chemotherapy with agents targeting the VEGF-A or EGFR pathways have not been demonstrated to increase survival in unselected gastric or gastroesophageal cancer patients (35-37). It is possible that Hedgehog pathway inhibition may have synergistic activity with these or other targeted agents.

In conclusion, we continue to move along toward an era of personalized medicine where we consider one cancer type like gastric cancer as composed of multiple different subtypes. The pathways driving tumor progression and metastasis can be quite different among these subtypes and thus response to targeted treatment is quite variable. The identification of response biomarkers is thus critical for targeted
Hedgehog signaling in CD44(+) gastric cancer cells

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treatments. In this study, we define a subgroup of CD44(+) cells in three gastric cancer cell lines with properties quite different from unselected cancer cells including the property of chemotherapy resistance. Hedgehog signaling pathway is important in the maintenance of these CD44(+) cells, and Hedgehog inhibition acts to reverse chemotherapy resistance in these cells. Although similar results were obtained in all three gastric cancer cell lines, we see from our correlative science studies of a clinical trial that the combination of Hedgehog inhibition and chemotherapy may only be beneficial in a minority of gastric cancer patients whose tumors express high levels of CD44.

ACKNOWLEDGMENTS

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REFERENCES


Hedgehog signaling in CD44(+) gastric cancer cells


Hedgehog signaling in CD44(+) gastric cancer cells


**Table 1: CD44 score, progression, and survival**

<table>
<thead>
<tr>
<th>Status</th>
<th>N</th>
<th>%</th>
<th>CD44 score mean±SD</th>
<th>p value</th>
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</thead>
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<tr>
<td><strong>All patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No progression</td>
<td>27</td>
<td>27.8</td>
<td>35.7±56.6</td>
<td>0.473</td>
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<tr>
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<td>70</td>
<td>72.2</td>
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</tr>
<tr>
<td>Alive</td>
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<td>26.4</td>
<td>44.4±56.8</td>
<td>0.804</td>
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<tr>
<td>Dead</td>
<td>67</td>
<td>73.6</td>
<td>40.7±64.4</td>
<td></td>
</tr>
<tr>
<td><strong>Chemotherapy only</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No progression</td>
<td>15</td>
<td>29.4</td>
<td>20.7±29.9</td>
<td>0.011</td>
</tr>
<tr>
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<tr>
<td>Alive</td>
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<td>25.0</td>
<td>24.2±28.7</td>
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<tr>
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<td>36</td>
<td>75.0</td>
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<tr>
<td><strong>Chemotherapy plus</strong></td>
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<tr>
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Hedgehog signaling in CD44(+) gastric cancer cells  Yoon C et al. -- 29

FIGURE LEGEND

Figure 1. Gastric cancer cells grown as spheroids show upregulation of CD44 and Hedgehog pathway proteins. A. Photos of gastric cancer cell lines AGS, MKN-45, and N87 and pancreatic cancer cell lines CFPAC-1 and PANC-1 grown as spheroids. B. Immunofluorescence photos of gastric cancer cells in regular media (monolayer) or in spheroid formation media at specified time points. Cells stained for CD44 (green), Gli1 (red), and DAPI (blue). C. Western blot showing expression of Hedgehog pathway proteins Sonic hedgehog (Shh), Patch 1 (Ptch1), Smoothened (Smo), and Gli1 in cell lines grown as monolayer cells versus as spheroids. D. Western blot showing expression of self-renewal proteins Sox2, Oct-4, and Nanog in cell lines grown as monolayer cells versus as spheroid.

Figure 2. Hedgehog pathway inhibition with Smo shRNA or vismodegib blocks spheroid formation. A. Western blot demonstrating knockdown of Smo in gastric cancer cell lines AGS, MKN-45, and N87 following transduction with Smo shRNA (Smo.shRNA) lentivirus compared to scrambled shRNA control (Scr.shRNA) lentivirus. B. Immunofluorescence photos for CD44 (green) and nuclei (blue) of gastric cancer cell lines treated with Smo.shRNA or Scr.shRNA and grown in spheroid formation conditions or treated with vismodegib (10 μM) or carrier (DMSO) and grown in spheroid formation conditions. C. Single cell assay of spheroid cells showing diameter of spheroids at selected time points following treatment with Smo.shRNA vs. Scr.shRNA or vismodegib (Vis, 10 μM) vs. carrier (DMSO). Bars represent standard deviation.
Figure 3. CD44(+) gastric cancer cells demonstrate chemotherapy resistance, which can be reversed with Hedgehog pathway inhibition. A. Immunofluorescence images of CD44 (green) and nuclei (blue) for CD44(+) and CD44(-) cells following magnetic bead sorting of AGS, MKN-45, and N87 gastric cancer cell lines. B. Western blot showing expression of Hedgehog pathway proteins Sonic hedgehog (Shh), Patch 1 (Ptch1), Smoothened (Smo), and Gli1 in CD44(+) and CD44(-) cells. Proliferation assay for CD44(+) and CD44(-) cells (C) and spheroid cells and monolayer cells (D) following treatment with 5-fluorouracil (5-FU) or cisplatin chemotherapy and vismodegib (Vis, 10 μM) compared to DMSO. Bars represent standard deviation.

Figure 4. Gastric cancer spheroid cells demonstrate increased migration, colony formation, and anchorage independent growth, which can be attenuated by Hedgehog pathway inhibition. A. Photos and graphs of migration assay of gastric cancer monolayer cells and spheroid cells treated with Smo shRNA (Smo.shRNA) compared to control (Scr.shRNA) or vismodegib (10 μM) compared to DMSO. B. Colony formation assay of gastric cancer monolayer cells and spheroid cells treated with Smo shRNA (Smo.shRNA) compared to control (Scr.shRNA) or vismodegib (10 μM) compared to DMSO. C. Photos and graph of soft agar assay of gastric cancer monolayer cells and spheroid cells treated with Smo shRNA (Smo.shRNA) compared to control (Scr.shRNA). Bars represent standard deviation.
Figure 5. Cisplatin chemotherapy combined with Hedgehog pathway inhibition with Smo shRNA synergistically block growth of MKN-45 gastric cancer xenografts. A. Proliferation of MKN-45 cells transduced with Smo shRNA (Smo.shRNA) or control (Scr.shRNA), and tumor growth curves for MKN-45 xenografts treatment with Scr.shRNA or Smo.shRNA and PBS or cisplatin. Immunohistochemical analysis of tumors for proliferation using PCNA immunohistochemistry (B), apoptosis using TUNEL immunofluorescence (C), and CD44 expression using CD44 immunohistochemistry (D). Bars represent standard deviation. *p<0.05 compared to control. **p<0.05 compared to all other groups.

Figure 6. CD44 may be a response biomarker in advanced gastric and gastroesophageal cancer patients treated with chemotherapy with or without vismodegib. A. Schema of the clinical trial. B. CD44 immunohistochemistry of patient tumor samples showing low, intermediate, and high CD44 expression. C. Kaplan-Meier overall survival curves for patients receiving chemotherapy alone and patients receiving chemotherapy plus vismodegib stratified by low and high CD44 score.
Yoon et al. Figure 1

A

Gastric Cancer Spheroids

<table>
<thead>
<tr>
<th>AGS</th>
<th>MKN-45</th>
<th>N87</th>
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Pancreatic Cancer Spheroids

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B

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<tr>
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Time in Spheroid Formation Media

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C

<table>
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<tr>
<th>AGS</th>
<th>MKN-45</th>
<th>N87</th>
<th>CFPAC-1</th>
<th>PANC-1</th>
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</thead>
</table>

| Monolayer | Spheroid | Monolayer | Spheroid | Monolayer | Spheroid | Monolayer | Spheroid | Monolayer | Spheroid |

<table>
<thead>
<tr>
<th>Shh</th>
<th>Ptch1</th>
<th>Smo</th>
<th>Gli1</th>
<th>β-actin</th>
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D

<table>
<thead>
<tr>
<th>AGS</th>
<th>MKN-45</th>
<th>N87</th>
</tr>
</thead>
</table>

| Monolayer | Spheroid | Monolayer | Spheroid | Monolayer | Spheroid |

| Sox2 | Oct-4 | Nanog | β-actin |
Figure 5

A. Number of cells and tumor volume over time. The graph shows the number of cells (top) and tumor volume (bottom) for different treatments over 17 days after the start of treatment. The treatments include Scramble shRNA + PBS, Smo shRNA + PBS, Scramble shRNA + Cisplatin, and Smo shRNA + Cisplatin. The bars indicate the number of cells, and the lines with error bars represent the tumor volume. Asterisks (*) indicate significant differences.

B. PCNAPositive cells (% of control). The graph shows the percentage of PCNA-positive cells for each treatment condition. The treatments are Scramble shRNA + PBS, Smo shRNA + PBS, Scramble shRNA + Cisplatin, and Smo shRNA + Cisplatin. Asterisks (*) indicate significant differences.

C. Number of apoptotic cells. The graph shows the number of apoptotic cells for each treatment condition. The treatments are Scramble shRNA + PBS, Smo shRNA + PBS, Scramble shRNA + Cisplatin, and Smo shRNA + Cisplatin. Asterisks (*) indicate significant differences.

D. CD44Positive cells (% of control). The graph shows the percentage of CD44-positive cells for each treatment condition. The treatments are Scramble shRNA + PBS, Smo shRNA + PBS, Scramble shRNA + Cisplatin, and Smo shRNA + Cisplatin. Asterisks (*) indicate significant differences.
Yoon et al. Figure 6

A

Advanced Gastric or GE Junction Adenocarcinoma

Randomize

FOLFOX chemotherapy + placebo

FOLFOX chemotherapy + Vismodegib

(n=124)

B

CD44 low

CD44 intermediate

CD44 high

C

Chemotherapy only

Chemotherapy + Vismodegib

Overall Survival

Overall Survival

n=28

n=20

p=0.455

n=15

n=28

p=0.168

Months

Months

0 10 20 30 40 50

0 10 20 30 40 50

High

Low

High

Low

p=0.455

p=0.168
Clinical Cancer Research

CD44 expression denotes a subpopulation of gastric cancer cells in which Hedgehog signaling promotes chemotherapy resistance

Changhwan Yoon, Do Joong Park, Benjamin Schmidt, et al.

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