Lentivirus-mediated CD44s expression increases human basal cell carcinoma cell resistance against vismodegib

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Abstract. High drug resistance, which is usually mediated by drug resistance-associated genes, is a characteristic of human tumours. CD44s, an ATP-binding cassette multidrug resistance transporter, is expressed in a variety of human cancers. In the present study, the effect of CD44s expression was investigated on BCC resistance against vismodegib. Lentiviral vectors were constructed to allow efficient CD44s expression. Cell clones expressing the CD44s construct were selected and expanded and then identified using qRT-PCR and western blotting. A lentiviral vector containing a blank sequence was used as a control. Cellular growth capacity and cell sensitivity to vismodegib were detected by MTT and Transwell assays, respectively. BCC cell growth was evaluated *in vivo* with a transplanted BCC nude mouse model. The cell clones expressing CD44s at high levels were identified by qRT-PCR and western blotting, and the difference in the cell proliferation rate between these cells and LV-CON BCC cells was assessed by growth curve analysis. The *in vitro* study revealed that treatment with vismodegib decreased BCC cell growth and migration; however, these effects were reversed by LV-CD44s overexpression. The *in vivo* study revealed that BCC tumour growth was significantly increased in nude mice transplanted with cells stably infected with CD44s compared with nude mice transplanted with cells infected with a control vector. Our investigation demonstrated that lentivirus-mediated CD44s expression may reverse the effects of vismodegib treatment on BCC.

Introduction

Basal cell carcinoma (BCC) is the most common skin malignancy. Cancer registries do not collect data on this skin cancer, so its prevalence and incidence are difficult to estimate. According to the American Cancer Society, in 2006, >2 million people were treated for cases of non-melanoma skin cancer (NMSC), the majority of which were BCC (1). The lifetime risk of developing skin cancer is estimated to be 1 in 5, and >97% of patients who develop skin cancer will have NMSC (2). BCC accounts for 75% of all skin cancers and is the most common skin malignancy. The incidence of BCC is rising by 3-8% each year (3); thus, the average lifetime risk that a Caucasian individual will develop BCC is 30% (4-6). BCC rarely metastasizes (7) but may cause extensive local tissue destruction when left untreated (8,9). Hence, BCC is becoming a serious health problem. Simple and cost-efficient medical treatments are clearly required. A thorough understanding of BCC pathobiology is required to develop such treatments. Considerable progress has been made with respect to the understanding of BCC pathobiology during the past few years.

BCC is characterized by abnormalities in the Hedgehog (Hh) signalling pathway that result in constitutively active Hh signalling. BCC tumours typically harbour an inactivating mutation of the tumour suppressor patch (Ptch) gene or an activating mutation in the smoothened (Smo) gene (10). Molecular studies have revealed that the Ptch gene is a human homologue of the Drosophila patched gene (11,12). Now known as PTCH, the gene encodes a receptor for the Sonic hedgehog (Shh) pathway, which is important for patterning and growth during vertebrate development (13). The Shh ligand binds to and inhibits the PTCH receptor to allow signalling through the Shh pathway. As an inhibitory protein, PTCH allows overactivation of the Shh pathway in cases in which it has inactivating mutations. Recent animal studies have revealed that mice overexpressing Shh in the context of normal PTCH expression and activity develop multiple BCCs and features of NBCCs (14,15). Activating somatic mutations in SMO, a seven-transmembrane protein immediately downstream of PTCH, were found in a selection of sporadic BCCs, and transgenic mice overexpressing mutant SMO have been revealed to develop skin abnormalities similar to those of BCCs (16). These findings indicate that SMO serves as a proto-oncogene. Overexpression of GLI proteins, transcription factors activated by SMO, in mouse models has been revealed to induce BCCs (17,18). Furthermore, continued Shh signalling has been revealed to be required for BCC carcinogenesis. A previous study demonstrated that mice engineered to conditionally

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express GLI-2 exhibited BCC regression when GLI-2 expression was inactivated (19). All of these studies support the concept that Shh signalling overactivation is necessary and perhaps sufficient for BCC development.

CD44 is a transmembrane cell-adhesion glycoprotein that participates in and regulates many cellular processes, including cellular growth, survival, differentiation, lymphocyte homing, and motility (20,21). CD44 affects a variety of cellular processes, most likely since multiple isoforms of the protein are produced by alternative splicing (22-24). CD44s, the smallest (standard) form of CD44 (CD44s), weighs approximately 80-95 kDa and lacks all variable CD44 exons. In breast cancer, cells undergoing EMT exhibit increased CD44 expression and TISC characteristics (25-27). CD44 expression has been described within TISC populations; however, the isoform responsible for its TISC characteristics remains unknown (21). CD44s, which is ubiquitously expressed in epithelial tissues, is the predominant CD44 variant and has recently been proposed to be essential for epithelial-to-mesenchymal transition (EMT) (28). Recent studies have demonstrated that the RNA-binding protein IMP3 stabilizes CD44 mRNA to facilitate cell migration and, more importantly, that CD44s combined with IMP3 can serve as a predictive biomarker for HCC (29). Collectively, the findings of these studies indicate that CD44s plays an important role in HCC progression.

To date, there is no evidence revealing that a relationship exists between CD44s expression and vismodegib resistance in BCC. In the present study, a CD44-harbouring lentiviral vector was used to express CD44s in a BCC cell line and then vismodegib cytotoxicity was evaluated in the cell line. Additionally, an *in vivo* model was used to evaluate the efficacy of vismodegib in mice implanted with tumours.

Materials and methods

Cell lines. The BCC cells, used in the present study, were isolated from BCC tumors that arose in irradiated Patched 1 (*Ptch1*)$^{+/-}$ mice, according to the protocol developed by So *et al* (30). The BCC cell lines were cultured in Gibco; Thermo Fisher Scientific, Inc., M154F media supplemented with 2% chelexed heat-inactivated foetal bovine serum, 1% penicillin-streptomycin, and 0.05 mM calcium chloride. Silibinin, carboxymethylcellulose (CMC), Harris haematoxylin, dimethyl sulfoxide (DMSO), and trypan blue were obtained from Sigma Aldrich; The human embryonic kidney cell line 293FT [American Type Culture Collection (ATCC), Rockville, MD, USA] was grown in DMEM supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences). All cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

Construction of the recombinant lentiviral vector encoding the CD44s gene. The sequences of the primers for the whole-length human CD44s cDNA sequence were as follows: sense, 5'-GCGTCGACATGGACAAGTTTTGGTGGCACG CAGCCTG-3' and antisense, 5'-CGGGATCCTTACACC CCAATCTTCATGTCCAC-3'. Briefly, the CD44s gene was amplified by PrimeSTAR® GXL DNA Polymerase (Takara Bio, Inc.) using BCC cell cDNA as a template, and then the CD44s gene and pLenti vector were digested by the enzymes *Sal*I and *BamH*I. Following recycling electrophoresis, the CD44s gene was subcloned into the pLenti plasmid, and the resulting recombinant plasmid, pLenti-CD44s, was identified by sequencing demonstrating its successful construction. Primer synthesis and DNA sequencing were performed by Shanghai Shangon Co., Ltd.. The viral particles were generated by the co-transfection of 293FT cells (American Type Culture Collection) with pLenti-CD44s or pLenti-CON and two packaging vectors via calcium phosphate-mediated transfection. Three days after transfection, the cell culture supernatants were harvested (2,000 x g for 5 min), filtered through filters with 0.45-μm pores and concentrated 100-fold by ultracentrifugation at 7,000 x g for 16 h. The viral particles were then stored in small aliquots at -80˚C.

Detection of CD44s mRNA levels in BCC cells by quantitative RT-PCR. CD44s mRNA expression in BCC cells infected with LV-CD44s was compared with that in BCC cells infected with LV-CON. Total RNA was isolated by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. CD44s mRNA expression levels were detected by quantitative RT-PCR (qRT-PCR) using a LightCycler 480 Instrument (Roche Diagnostics) and a SYBR® Premix Ex Taq™ kit (Takara Bio, Inc.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a normalizing control. The specific sequences of these PCR primers, which were designed by Primer premier 5.0 software (Premier Biosoft International), were as follows: CD44s forward, 5'-GGAGCAGCACTTCAGGAGGTTAC-3' and reverse, 5'-GGAATGTGTCTTGGTCTCTGGTAGC-3'; and GAPDH forward, 5'-TCATGGGTGTGAACCATGAGAA-3' and reverse, 5'-GGCATGGACTGTGGTCATGAG-3'. Cycling was performed under the following conditions: 95˚C for 10 min (to activate DNA polymerase), followed by 40 cycles of 95˚C for 15 sec, 55˚C for 20 sec, and 72˚C for 10 sec. The specificity of the amplification products was confirmed by melting curve analysis. Independent experiments were performed in triplicate. The relative mRNA expression levels of the samples were normalized against the mRNA expression level of GAPDH. The cycle threshold (Cq) value was the output from the instrument software, and the relative expression level was calculated using $2^{-\Delta\Delta Cq}$ method according to the following formula: $\Delta\Delta$Cq (target gene)=Cq (target gene)-Cq (control gene) (31).

Detection of target protein expression by western blotting. Protein was extracted from the cultured cells with RIPA lysis buffer (1% NP40, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonyl fluoride, and 0.5% sodiumdeoxycholate in PBS) containing a proteinase inhibitor (Roche Diagnostics) on ice for 30 min. The supernatants were collected by centrifugation at 12,000 x g for 20 min at 4˚C, and the protein concentrations were determined using a BCA assay kit (Pierce Biotechnology; Thermo Fisher Scientific, Inc.). Twenty micrograms of protein mixed with 2xSDS loading buffer [125 mM Tris-HCl, 4% SDS, 20% glycerol, 100 mM dithiothreitol (DTT), and 0.2% bromophenol blue] was loaded into each lane and separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto polyvinylidenedifluoride membranes (PVDF; EMD Millipore), which were blocked with 5% non-fat dry milk in TBST (20 mM Tris-HCl, pH 7.5;

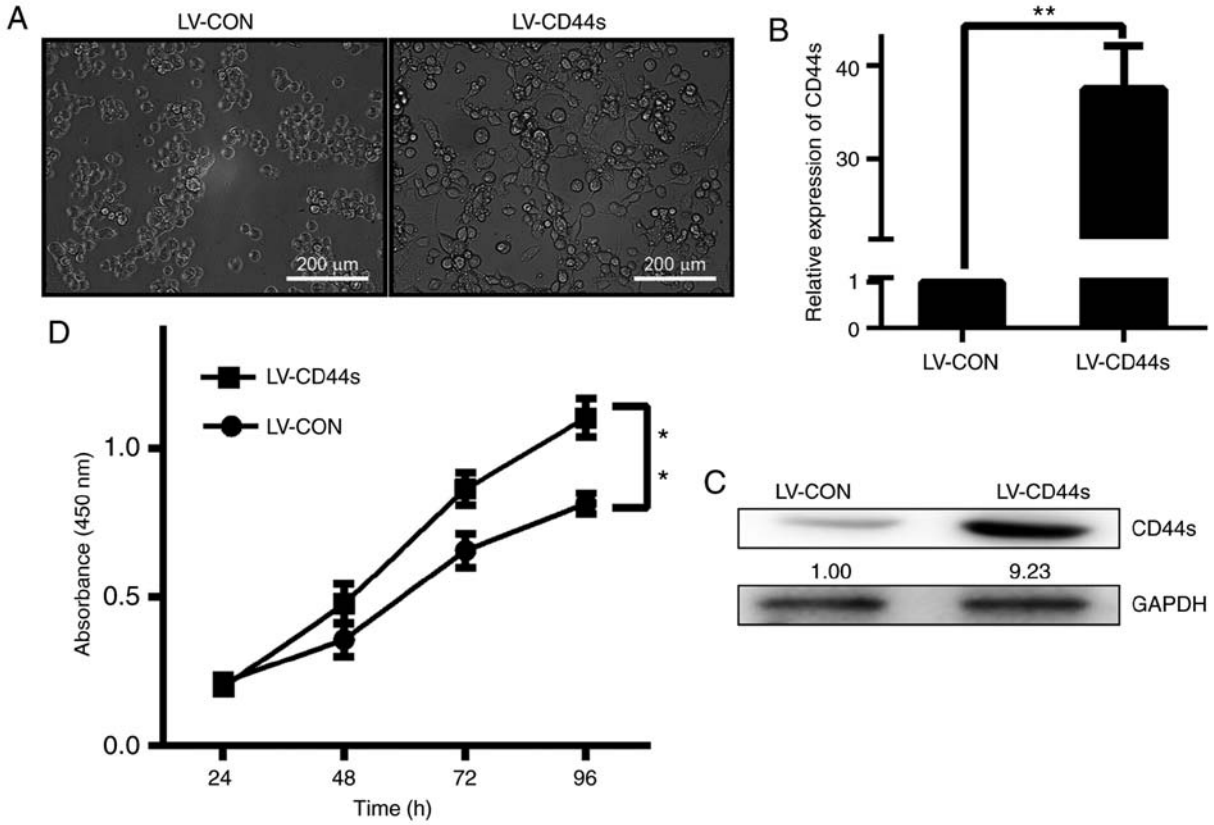

Figure 1. CD44s-containing lentiviral vector (LV-CD44s) construction and CD44s mRNA and protein expression in BCC cells. (A) CD44s-containing lentiviral vector (LV-CD44s) construction and infection of BCC cells. (B) CD44s mRNA expression in BCC cells, as determined by quantitative RT-PCR. n=3, **P<0.01 vs. LV-CON. (C) Cellular growth curve. Cell growth and viability over 96 h were assessed by MTT assay. (D) Analysis of CD44s expression levels, as detected by western blotting, in BCC cells treated with the CD44s construct (LV-CD44s) and the control construct (LV-CON). Data were expressed as the mean ± SD. n=3, **P<0.01 vs. LV-CON.

150 mM NaCl; 0.1% Tween-20) for 2 h at room temperature. The membranes were then incubated with antibodies against Shh (dilution 1:500; cat. no. ab53281), Smo (dilution 1:1,000; cat. no. ab113438) and Ptch1 (dilution 1:800; cat. no. ab53715; all from Abcam, Cambridge, UK) or GAPDH (dilution 1:2,000; cat. no. 51332; Cell Signaling Technology, Inc.) overnight at 4˚C. Finally, the membranes were washed and then incubated with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (dilution 1:5,000; cat. no. 7076) or HRP-conjugated goat anti-rabbit IgG (dilution 1:5,000; cat. no. 7074; both from Cell Signaling Technology, Inc.) for 2 h at room temperature. The resulting protein bands were detected using enhanced chemiluminescence (ECL) luminol reagent (EMD Millipore). GAPDH was used to detect target protein level modulation, and the images were analysed by Image-Pro® Plus software (Media Cybernetics).

Apoptosis assay. Approximately 1x10^6 cells were centrifuged at 2500 x g for 5 min, washed with cold BioLegend Cell Staining Buffer, and then resuspended in Annexin V Binding Buffer. Approximately 100 μl of cell suspension was transferred into a 5-ml test tube, to which 5 μl of FITC Annexin V and 5 μl of 7-ADD Viability Staining Solution were subsequently added. The cells were then gently vortexed and incubated in the dark for 15 min at room temperature. The volume of the solution was increased to 500 μl with Annexin V Buffer and analysed with a FACS Calibur Flow Cytometer (BD Biosciences).

Transwell assay. A Transwell assay was performed with a pre-coated cell invasion kit (pore size, 8.0 μm; Corning, Inc.). BCC cells (5x10^4/well) were allowed to migrate from the upper chamber to the lower chamber, which contained medium with 30% FBS. Following 60 h of incubation, the cells that had migrated through the membrane were stained with 0.1% crystal violet at room temperature for 20 min and then counted under a light microscope (6 random fields/well).

MTT detection. BCC cells were seeded in 96-well plates at a density of 5x10^3 cells/well. The cells were treated with various agents at 24 h after seeding. Following 48 h of incubation under normal culture conditions, the cells were treated with MTT at a final concentration of 5 mg/ml. Four hours later, DMSO (Sigma-Aldrich; Merck KGaA) was added to the wells to dissolve the crystals. The wells were shaken horizontally for 10 min. The OD value was assessed at 405 nm by a micro-plate reader (Bio-Rad Laboratories, Inc.). The IC_{50} value was determined based on the relative absorbance of MTT, which was determined by probit regression analysis with SPSS18.0 statistical software (SPSS, Inc.).

In vivo treatments. BCC cells were cultured in a 75-cm^2 flask, after which cell suspensions containing 2x10^6 cells were subcutaneously transplanted into 6-week-old athymic female BALB/c nude mice (~16 g per mouse). Each group was comprised of five mice. All the mice were maintained in

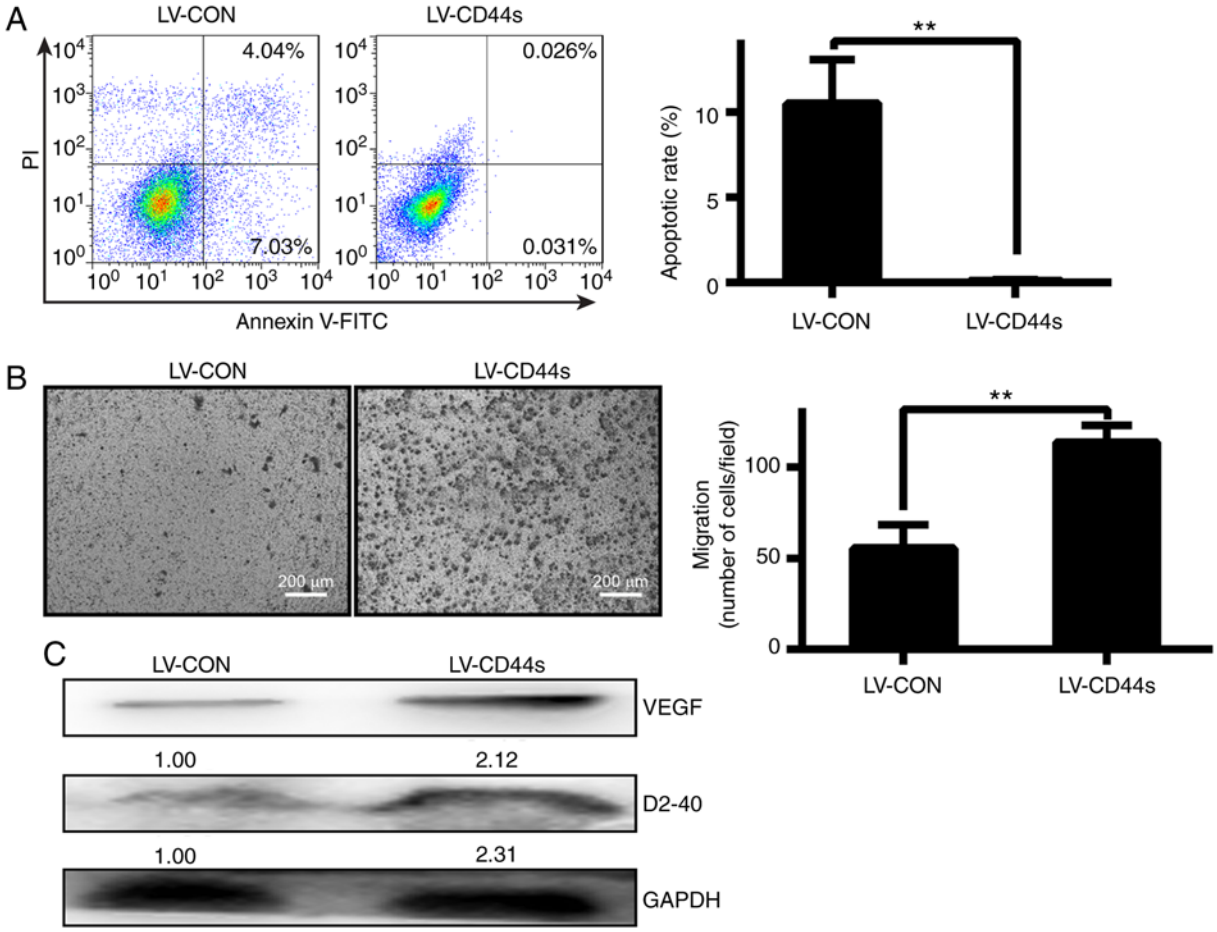

Figure 2. Upregulation of CD44s by LV-CD44s increased BCC cell migration. (A) FACS was used to assess apoptosis in BCC cells treated with the LV-CD44s construct and the LV-CON construct. Data were expressed as the mean ± SD. n=3, **P<0.01 vs. LV-CON. (B) The stained cells are those that migrated through the polycarbonate membrane to its lower surface (original magnification, x100). The migration assay indicated that LV-CD44s significantly increased BCC cell migration ability. The bars indicate the mean ± SD. **P<0.01 vs. LV-CON group. (C) VEGF and D2-40 protein expression was detected by western blotting in both groups.

caged housing in a specifically designed pathogen-free (SPF) isolation facility at 24˚C with a 12-h light/dark cycle, and fed rodent chow and water *ad libitum*. All the mice exhibited a 50-mm^3 tumour mass on day 7 after transplantation. The mice were divided into two groups, each of which was comprised of 5 animals. The animals in group 1, or the LV-CON group, received LV-CON treatment, and the animals in group 2, or the LV-CD44s group, received LV-CD44s treatment twice a week. The tumour volume was evaluated at day 6, 10, 12, 14, 16, 18, 20, 22 and 24 post-transplantation. Two orthogonal diameters of each tumour were measured with Vernier callipers. The tumour volume was calculated with the following formula: Tumor volume = (lengthxwidth^2)/2. Tumours with weights outside the range of 50-200 mg at the start of the treatment period were excluded from the study. On day 24 after transplantation, all the mice were euthanized by 20% of CO_2/min asphyxiation. The tumor masses were weighed using electronic scales. The tumours were collected and fixed in 10% formalin. Immunohistochemistry (IHC) was used to detect CD31 expression in tumour tissues. Relative tumour size (RTS) was calculated as the tumour volume at the time of measurement divided by the tumour volume at the time of treatment. The mean RTS value was plotted as a function of time for the different treatment groups. All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Xi'an Jiaotong University (No. 2016061).

Statistical analysis. Statistical analyses were performed using SPSS statistical software 18.0 (SPSS, Inc.). Normally distributed data are presented as the mean ± SD of at least three independent experiments. Student's t-tests or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were performed to assess statistical significance. The results were considered statistically significant at P<0.05.

Results

Lentivirus-mediated CD44s expression in BCC cells. CD44 was initially identified as a lymphocyte homing receptor and transmembrane glycoprotein that is commonly expressed in embryonic stem cells and haematopoietic and cancer stem cells (32,33). However, little research on the function of CD44s in BCC cells has been reported. It has been demonstrated that lentiviral-mediated gene expression facilitates effective, stable gene expression in multiple biological systems and assists in the elucidation of gene functions in numerous cell types. Lentiviruses containing CD44s constructs were used

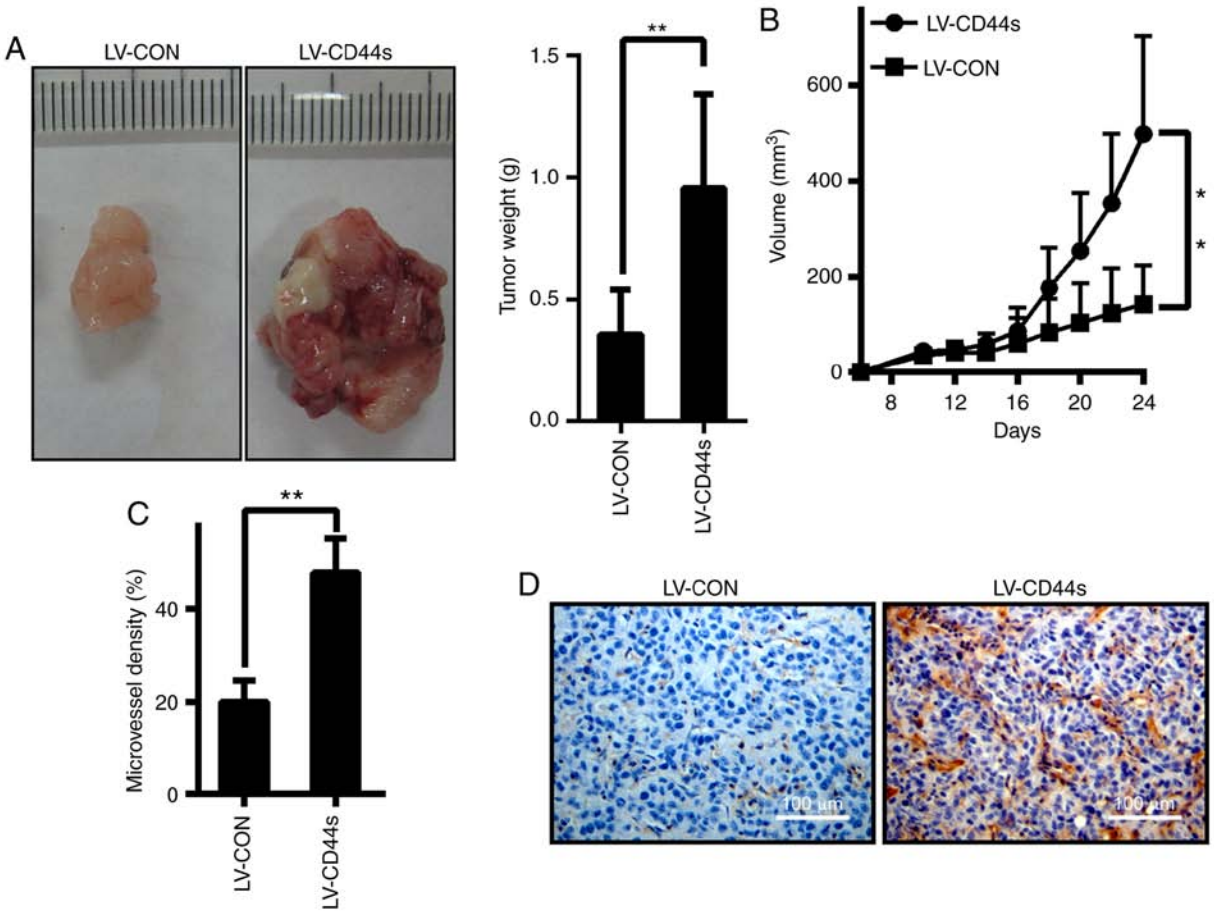

Figure 3. Overexpression of CD44s by lentiviruses increases BCC tumour growth in nude mice. (A) Comparison of the sizes and weight of the tumours in LV-CD44s and LV-CON mice. (B) Effects of CD44s on the growth of the tumours subcutaneously transplanted into nude mice. The effects were evaluated by calculating RTS. **P<0.01 vs. LV-CON group. (C) Analysis of MVD in mice after treatment. MVD was determined by counting the number of CD31-positive blood vessels per field in selected vascularized areas. The data were represented as the average MVD, which was determined from 10 randomly selected areas. Each bar represents the mean ± SD. **P<0.01 vs. LV-CON group. (D) Representative immunohistochemical analysis of CD31 staining on the tumor sections.

to express CD44s in BCC cells. Lentiviruses containing blank constructs (LV-CON)were used as controls (Fig. 1A). As revealed in Fig. 1A, compared with BCC control cells, CD44s overexpression cells exhibited a more mesenchymal phenotype with elongated shape and reduced cell-cell contact as observed by inverted microscopy. CD44s mRNA expression in BCC cells was compared with that in parental BCC cells and LV-CON cells by qRT-PCR. To confirm the expression of CD44s in the aforementioned cell clones, CD44s protein expression was detected by western blotting. It was revealed that CD44s expression, as demonstrated by qRT-PCR and western blotting, was higher in BCC cells infected with LV-CD44 than in LV-CON cells (Fig. 1B and C). After the CD44s construct (LV-CD44s) was transfected into BCC cells, we selected the cell clones stably expressing CD44s and cultured and analysed them separately. Cell proliferation was monitored for 96 h after the BCC cells were infected with LV-CD44s and LV-CON. The growth of the cells infected with LV-CD44s was markedly increased compared with that of the cells infected with LV-CON (Fig. 1D).

CD44s affects BCC cell apoptosis and proliferation in vitro and in vivo. CD44 is a transmembrane glycoprotein and is overexpressed in BCC, which suggests that it is required for cancer proliferation. First, BCC cells were successfully infected with a lentivirus (Fig. 1A). FACS assay revealed that the number of viable cells in the LV-CD44s group was significantly decreased compared with that in the LV-CON group (**P<0.01, Fig. 2A). A Transwell assay revealed that the migration potential of the cells was significantly increased when CD44s was overexpressed by LV-CD44s in BCC cells (**P<0.01, Fig. 2B). Furthermore, VEGFA and D2-40 expression in the LV-CD44s group was significantly increased compared with that in the LV-CON group(Fig. 2C). Based on these results, it was concluded that CD44s plays a critical role in BCC cell proliferation and migration potential.

To examine the effects of CD44s on tumour growth *in vivo*, BCC cells stably expressing control vectors or CD44s were inoculated into the subcutaneous tissue of BalB/C nude mice. All the animals developed tumours 7 days after inoculation. The tumours in the LV-CD44s group were significantly larger than those in the LV-CON group at 24 days after inoculation (Fig. 3A). The mean tumour volume in mice inoculated with BCC cells expressing LV-CON was 143.20±81.41 mm^3, while that in mice inoculated with BCC cells expressing LV-CD44s was 498.23±204.65 mm^3 (Fig. 3B). Given that angiogenesis is an integral component of BCC, it was explored whether CD44s

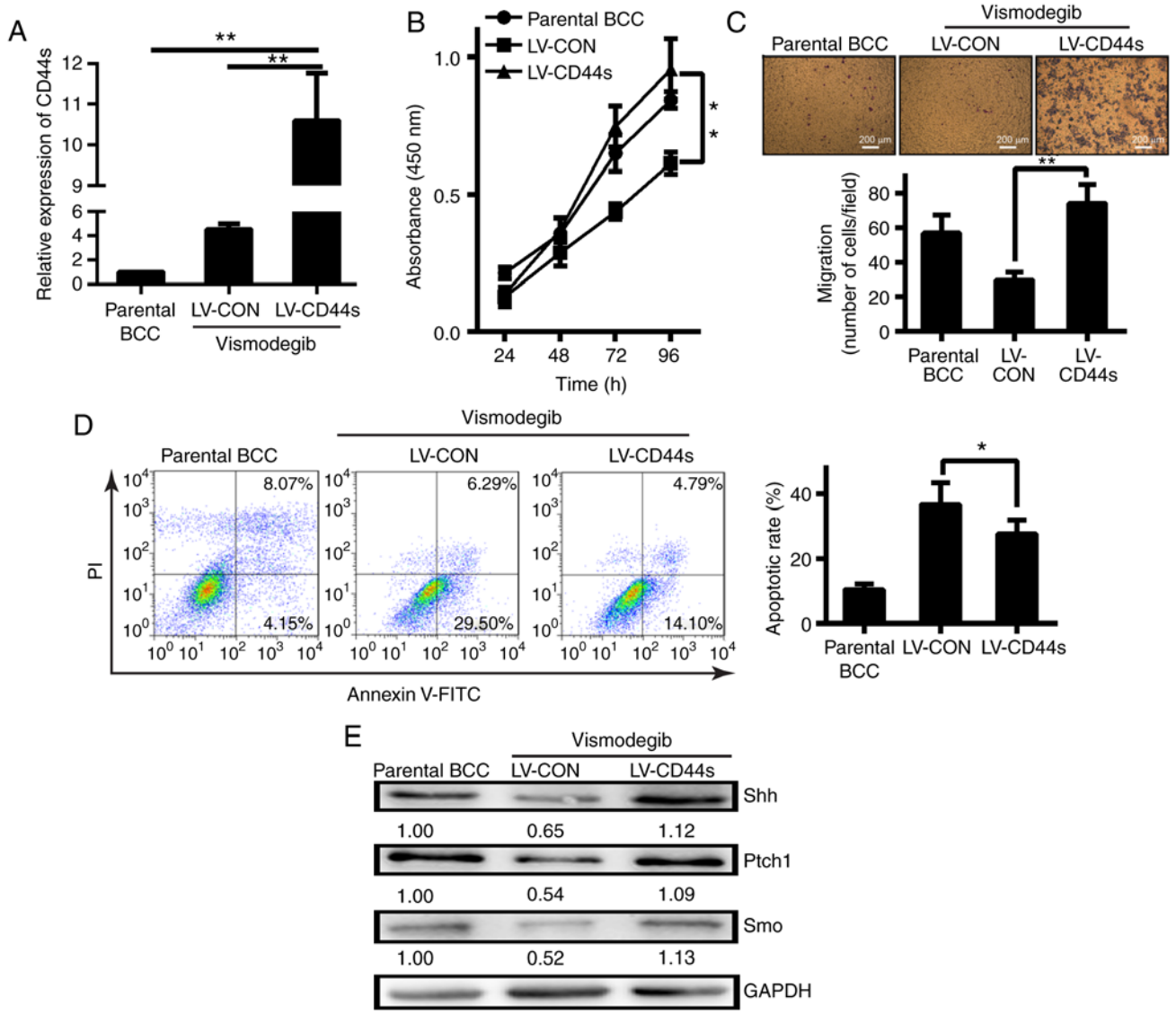

Figure 4. CD44-positive BCC cells demonstrate vismodegib resistance, which is signified by Hh pathway activation. (A) Detection of CD44s expression by real-time PCR. **P<0.01 vs. LV-CON or parental BCC cells. (B) MTT detection of BCC proliferation after vismodegib treatment *in vitro*. **P<0.01 vs. LV-CON. (C) Transwell detection of BCC migration after vismodegib treatment *in vitro*. **P<0.01 vs. LV-CON. (D) Number of apoptotic cells (Annexin V-positive cells) in a BCC cell population treated with vismodegib. *P<0.01 vs. LV-CON. (E) Western blotting revealing the expression of the Hh pathway proteins Shh, Ptch1 and Smo in parental BCC, LV-CD44s and LV-CON cells.

plays a role in BCC angiogenesis. Immunohistochemical analysis revealed that CD31 (a marker for microvessels denoting enhanced angiogenesis) levels were significantly higher in tumours in the CD44s group than in tumours in the LV-CON control group (Fig. 3C and D), suggesting that CD44s has angiogenic effects in BCC. These data demonstrated that CD44s plays an important role in promoting BCC cell growth *in vivo*.

Effects of the Hh pathway on BCC cells with CD44 expression. We next examined the effects of CD44 expression on BCC characteristics, including CD44s expression, BCC proliferation, migration and apoptosis, after vismodegib treatment. The BCC cell line expressing CD44 demonstrated greater expression (Fig. 4A) and proliferation after vismodegib treatment than the control or parental cell lines (Fig. 4B). Vismodegib treatment reduced BCC cell migration, while CD44s expression reversed this effect (Fig. 4C). BCC cell apoptosis was also assessed and it was revealed that more LV-CON cells than CD44-expressing BCC cells underwent apoptosis (Fig. 4D). CD44-positive cells revealed upregulated expression of the Hh pathway proteins Shh, Ptch1, and Smo compared with CD44-negative cells (Fig. 4E). Thus, CD44s-positive BCC cells had increased proliferation and migration abilities and underwent less apoptosis compared with CD44-negative cells (LV-CON). These properties are dependent on Hh signalling. The levels of the self-renewal proteins Oct2 and Nanog and other related stem cell markers were decreased in BCC cells lacking CD44 (LV-CON) after vismodegib treatment. However, the levels of these proteins remained upregulated in BCC cells with CD44s overexpression (Fig. 5). Thus, BCC cell growth was associated with sustained increases in the levels of CD44, Hh pathway proteins, and some self-renewal proteins.

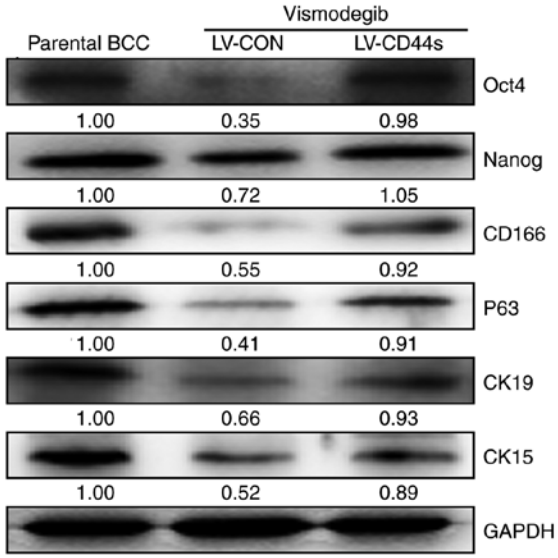

Figure 5. Western blotting revealing differences in the expression levels of the self-renewal proteins Nanog and Oct-4 and stem cell markers in parental BCC, LV-CD44s and LV-CON groups after vismodegib treatment.

Discussion

The present study is the first to demonstrate that the Hh signalling pathway plays a vital role in the maintenance of chemotherapy resistance in BCC cells with CD44 expression. BCC cells were grown with CD44 overexpression and it was revealed that this cell line displayed increased expression of Hh pathway proteins and certain self-renewal proteins. Inhibition of Hh signalling using vismodegib blocked BCC cell growth. BCC cells were highly sensitive to vismodegib therapy *in vitro*, and this therapy sensitivity was reversed by CD44 overexpression. Cells with CD44 overexpression displayed increased proliferation and migration, phenotypic features that were attenuated following Hh pathway inhibition with vismodegib.

The pivotal molecular abnormality in BCC carcinogenesis is aberrant Hh signalling pathway activation. The Hh signalling pathway was first described in genetic studies of embryonic segmentation and imaginal disk specification in Drosophila. It is highly conserved from insects to vertebrates, and vertebrates have multiple homologues of several components of the pathway (34). The following three homologues are found in mammals: Shh, Indian hedgehog (Ihh), and Desert hedgehog (Dsh). Shh is the most commonly expressed and best characterized homologue and is crucial for the nervous system, axial skeleton, lung, skin, hair, and stem cell population development and maintenance. Shh is synthesized as a 45-kDa precursor protein that is auto-catalytically cleaved and covalently modified by palmitate and cholesterol. Shh is secreted and binds to its receptor, Ptch1. Ciliary ablation was revealed to strongly inhibit the development of BCC and medulloblastoma when these tumours were driven by an activated form of the transmembrane protein SMO. Conversely, ciliary removal accelerated tumourigenesis induced by constitutively active GLI-2 (35,36).

CD44 is a transmembrane glycoprotein and the principal cell surface receptor for hyaluronic acid, a major component of the ECM (37). CD44 plays an important role in communication in cell-matrix interactions and also plays a role in cell motility, matrix degradation, proliferation, and survival. The major form of CD44 on epithelial cells is CD44s (standard), but some cells possess an isoform of CD44 known as CD44v (variant). Several studies have revealed that an association exists among CD44v6 expression, gastric cancer lymph node metastasis, and prognosis (38,39). In the present study, lentiviral vectors harbouring CD44s were used to overexpress the protein to analyse its function. CD44 expression was displayed by BCC cells with malignant transformation, a property that may be associated with Hh pathway activation. The prognostic value of the tumor microvascular density (MVD) in cancer has been examined in several studies, with correlations with tumor recurrence, disease-free or overall survival. Thus, the association with angiogenesis was assessed by measuring MVD. There are a few factors produced by MVD that are responsible for the induction of angiogenesis, with VEGF and D2-40, is thought to have a key role in it (40).

Vismodegib is the first oral medicine approved by the US Food and Drug Administration for the treatment of adults with advanced BCC (both locally advanced and distantly metastatic BCC) that has recurred after surgery or cannot be resected or irradiated. Vismodegib is a competitive antagonist of SMO, a component of the Hh signalling pathway. Total SMO inhibition causes the transcription factor GLI-1 to remain inactive, which in turn suppresses the expression of genes regulated by the Hh signalling pathway (41). However, vismodegib, which targets the Hh signalling pathway, has not been demonstrated to inhibit the growth of BCC cells with CD44s overexpression *in vitro*. It is possible that Hh pathway inhibition synergizes with CD44s expression. Vismodegib has not been demonstrated to inhibit the growth of BCC cells with lentivirus-mediated CD44s overexpression in nude mice *in vivo*. This will be addressed in a future study.

In the present study, we defined a subgroup of BCC cells comprised of CD44-positive cells that have properties that are very different from those of unselected cancer cells, including the property of chemotherapy resistance. The Hh signalling pathway is important in the maintenance of these CD44-overexpressing cells, and Hh inhibition acts to reverse therapy resistance in these cells. Similar results have been obtained in research involving other cancer cell lines. Our correlative scientific study revealed that the combination of Hh inhibition and oncogene molecular downregulation may be beneficial in only a minority of patients with BCC, namely, patients whose tumours express CD44 at low levels.

In conclusion, CD44s is highly expressed in BCC cells, as demonstrated by qRT-PCR and western blotting, and differences were observed between the proliferation rates of LV-CD44s BCC cells and those of LV-CON BCC cells by growth curve analysis. *In vitro*, vismodegib treatment reduced growth and migration in LV-CON BCC cells; however, LV-CD44s expression reversed these changes. *In vivo*, the growth of BCC tumours stably infected with CD44s constructs was significantly increased in the transplanted nude mouse model compared with that of BCC tumours comprised of control cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JR, XM and CT contributed to the study conception and design of the experiments. JR, XM, CT and ZL performed experiments. CT acquired data. JR and XM contributed with the statistical analysis of the data. CT and ZL participated in the writing of the manuscript. JR, XM, CT and ZL drafted, edited, critically revised and approved final version of manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Xi'an Jiaotong University (No. 2016061).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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