Lentivirus-mediated RNA interference inhibits the tumorigenicity of cluster of differentiation 44+ tumor cells in hypopharyngeal cancer

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Abstract. The present study aimed to investigate whether the inhibition of cluster of differentiation (CD)44 expression reduces the tumorigenicity of CD44+ cancer stem cells in hypopharyngeal cancer. To assess this, effective recombinant CD44 short hairpin RNA-expressing lentiviruses were produced. Lentivirus-mediated RNA interference (RNAi) was then used to knockdown CD44 gene expression in the hypopharyngeal cancer FaDu cell line. The viability of FaDu cells in the two control groups and the RNAi group (RNAi-CD44 lentiviral vector) was detected using an MTT assay *in vitro*. Cells from each group were injected into non-obese diabetic/severe combined immunodeficiency mice and their tumorigenicity determined *in vivo*. Following lentivirus-mediated RNAi, an MTT assay indicated that cells from the RNAi group exhibited lower viability than the control group. The *in vivo* tumorigenicity study further revealed a significant difference in tumorigenic rates between the RNAi group and the control group (Fisher's exact test, P<0.05). In addition, tumors in the RNAi group of animals had a longer incubation period than those in the control groups, and the mean tumor volume was also significantly smaller (t=3.47, P<0.05). Pathological study confirmed that all tumors were poorly differentiated squamous cell carcinomas with cellular heterogeneity. The viability of the hypopharyngeal cancer FaDu cells *in vitro* and their tumorigenicity *in vivo* were markedly inhibited once CD44 was knocked down. The results of the present study therefore suggest that CD44 may confer tumorigenic characteristics upon CD44+ cancer stem cells in hypopharyngeal cancer.

Introduction

Cancer stem cells, a small number of cells with the potential for unlimited self-renewal, have been recently theorized to play a substantial role in tumor growth and the promotion of tumor formation (1). Cancer stem cells have already been identified in numerous types of carcinoma, including brain neoplasms, leukemia and cancer of the breast, colon and lung (2-10). In malignant head and neck tumor stem cells, cluster of differentiation (CD)133 has been reported to be a biological marker of cancer stem cells in laryngeal cancer (11) and nasopharyngeal carcinoma (12); CD133, CD44 and aldehyde dehydrogenase 1 are all cancer stem cell markers in oral carcinoma (13,14). However, there have been few studies investigating the role of cancer stem cells in hypopharyngeal carcinoma. CD44+ cells from the hypopharyngeal cancer FaDu cell line were previously found to have a higher proliferative capacity and tumorigenic potential, which suggests that CD44+ hypopharyngeal tumor cells may be cancer stem cells, or that cancer stem cells in hypopharyngeal cancer exist in the CD44+ tumor cell population (15). To determine whether the CD44 gene serves a notable role in the cancer stem cell properties of CD44+ cells, the tumorigenicity of CD44+ cells was assessed in the FaDu cell line following the silencing of CD44 gene expression using recombinant lentiviral vectors that specifically knocked down the expression of CD44. The viability of FaDu cells *in vitro* and their tumorigenicity *in vivo* were subsequently examined to determine whether CD44 confers the biological characteristics of cancer stem cells in CD44+ cells in hypopharyngeal cancer.

Materials and methods

Design and construction of CD44 short hairpin RNA (shRNA)-expressing lentivirus. The hU6-MCS-CMV-EGFP-iRNA plasmid (Shanghai Genechem Co., Ltd., Shanghai, China) was used to generate the CD44 shRNA-expressing lentivirus.

According to The National Center for Biotechnology Information GenBank (https://www.ncbi.nlm.nih.gov/genbank/) published human CD44 mRNA (NM_00100139) sequence information and RNA interference sequence design principles, four interfering target sequences were designed (Table I). Subsequently, target sequences were used to carried out BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison in human genome data to exclude the other similar nucleotide sequences. For each targeting sequence, two oligonucleotides containing the sense and antisense of the targeting sequences (with a loop sequence in between) were synthesized (Table II). The two oligonucleotides were resolved together in water at 90°C for 15 min and then placed at room temperature to cool down and form double-stranded DNA. The double-stranded DNA was ligated into the hU6-MCS-CMV-EGFP-iRNA plasmid digested with *Age*I and *Eco*RI restriction endonucleases (New England Biolabs, Inc., Ipswich, MA, USA) to generate the CD44 shRNA-expressing lentiviral vectors: LV-GFP-CD44-shRNA-1, LV-GFP-CD44-shRNA-2, LV-GFP-CD44-shRNA-3 and LV-GFP-CD44-shRNA-4. The ligated vectors were transformed into competent *Escherichia coli* DH5α cells (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and a single colony was selected for PCR amplification. The reaction mixture volume of 20 μl, comprising 0.4 μl upstream and downstream primers (upstream: 5'-CCATGATTCCTTCATATTTGC-3'; downstream: 5'-CGCGTGGATAACCGTATTAC-3'), 15.2 μl ddH$_2$O, 2 μl 10X buffer (Takara Bio Inc., Japan), 0.8 μl 2.5 mM dNTP Mixture (Takara Bio Inc., Otsu, Japan), 0.2 μl Taq DNA polymerase (Takara Bio Inc., Japan), and 1 μl 10 ng/μl template. The PCR reaction thermocycler conditions were as follows: Pre-denaturing at 94°C for 30 sec, denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, extending at 72°C for 40 sec, then 72°C for a final 6 min. The total PCR process was 30 cycles. Reactions were performed with the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, Inc.). The QIAGEN Plasmid Midi Kit (Qiagen GbmH, Hilden, Germany) was used to extract the plasmid, which was then sent to Shanghai Genechem Co., Ltd., (Shanghai, China) for nucleotide sequencing to verify the correct constructs (16). 293T cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Recombinant lentiviruses were produced by co-transfecting 293T cells with the recombinant lentiviral vector and packaging plasmids (Shanghai Genechem Co., Ltd.) (pHelper 1.0, including gag/pol, and pHelper 2.0, including vesicular stomatitis virus G) using the cationic lipid complex method (Lipofectamine 2000; Invitrogen; Thermo Fisher Scientific, Inc.). The culture supernatants containing the produced viruses were harvested 48 h after transfection and concentrated by centrifugation at 4,000 x g at 4°C for 10-15 min. Aliquots of the concentrated viruses were stored at 80°C for subsequent use. The infectious titer was measured using a proportional dilution method with 293T cells and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. Total RNA from 293T or FaDu cells was extracted using TRIzol® (Takara Bio Inc.), and reversed transcribed according to the manufacturer's protocol. cDNA samples were

Table I. Sequence of CD44 siRNA group.

CD44-knockdown siRNA group	Sequence	GC content, %
KD1	CCTCTGCAAGGCTTTCAAT	47.37
KD2	GCTCTGAGCATCGGATTTG	52.63
KD3	GCATCGGATTTGAGACCTG	52.63
KD4	GGCTTTCAATAGCACCTTG	47.37

CD, cluster of differentiation; siRNA, short interfering RNA; KD1, CD44-knockdown by siRNA 1.

amplified using SYBR® Premix Ex Taq™ (Takara Bio Inc.). The primer sequences were used as follows: CD44: 5'-AGGCTGAGACAGGAGGTTA-3' (forward); 5'-CCTCCCTTATTTCTATCGTG-3' (reverse); GAPDH: 5'-GGTGAAGGTCGGTGGAACG-3' (forward); 5'-CTCGCTCCTGGAAGATGGTG-3' (reverse). The PCR reaction conditions were as follows: 95°C pre-denaturing for 30 sec, 95°C denaturing for 5 sec, 60°C annealing for 34 sec for 40 cycles, then 95°C denaturing for 15 sec, 60°C annealing for 60 sec, and 95°C for a final 15 sec. Reactions were performed with the ABI PRISM 7500 System (Thermo Fisher Scientific, Inc.). Gene expression in each sample was normalized to GADPH expression. Statistical analyses were performed on qPCR results obtained through the $2^{-\Delta\Delta Cq}$ method (17). The experiment was repeated in triplicate.

Screening of shRNA-expressing lentiviruses. FaDu cells were subcultured at a density of 1x10⁶ cells per well into 6-well tissue culture plates. The experiment was divided into a low multiplicity of infection (MOI) group and a high MOI group. There were also six subgroups: A blank control group [CON, infected with PBS (0.01 mol/l) alone]; a negative virus control group [NC, infected with the negative control lentiviral vector (8E + 8 TU/ml)]; and four RNAi groups (KD1, infected with the shRNA1-CD44 lentiviral vector; KD2, infected with the shRNA2-CD44 lentiviral vector; KD3, infected with the shRNA3-CD44 lentiviral vector; and KD4, infected with the shRNA4-CD44 lentiviral vector; all shRNA sequences are shown in Table II). Following incubation at 37°C for 48 h of culture, cells were infected with specific or negative control lentiviral vectors, at the aforementioned MOI. Cells were examined by fluorescence microscopy 72 h after lentiviral transduction. On day 5 after transduction, the cells were harvested to determine the efficiency of CD44 silencing by RT-qPCR. The cells were firstly washed with PBS and digested with 0.25% trypsin at 37°C for 3 min. RPMI-1640 medium (1 ml) with 10% FBS to stop digestion was then added, followed by centrifugation at 500 x g for 5 min at 28°C . In the RNAi group, the most efficient shRNA-CD44 lentiviral vector (hereafter termed KD) was used in the following *in vitro* and *in vivo* experiments.

Cell culture. The FaDu and 293T cells, obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute

Table II. Sequences of oligonucleotides for constructing recombinant lentiviral vectors expressing shRNAs.

shRNA group	5'	STEMP	Loop	STEMP	3'
KD1 sense	CCGG	GACCTCTGCAAGGC TTTCAAT	CTCGAG	ATTGAAAGCCTTGCAG AGGTC	TTTTTG
KD1 antisense	AATTCAAAAA	GACCTCTGCAAGGC TTTCAAT	CTCGAG	ATTGAAAGCCTTGCAG AGGTC	
KD2 sense	CCGG	AAGCTCTGAGCATC GGATTTG	CTCGAG	CAAATCCGATGCTCAG AGCTT	TTTTTG
KD2 antisense	AATTCAAAAA	AAGCTCTGAGCATC GGATTTG	CTCGAG	CAAATCCGATGCTCAG AGCTT	
KD3 sense	CCGG	GAGCATCGGATTTG AGACCTG	CTCGAG	CAGGTCTCAAATCCGA TGCTC	TTTTTG
KD3 antisense	AATTCAAAAA	GAGCATCGGATTTG AGACCTG	CTCGAG	CAGGTCTCAAATCCGA TGCTC	
KD4 sense	CCGG	AAGGCTTTCAATAG CACCTTG	CTCGAG	CAAGGTGCTATTGAAA GCCTT	TTTTTG
KD4 antisense	AATTCAAAAA	AAGGCTTTCAATAG CACCTTG	CTCGAG	CAAGGTGCTATTGAAA GCCTT	

shRNA, short hairpin RNA; KD1, CD44-knockdown by shRNA 1; STEMP, sequences of oligonucleotides.

for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), were recovered from frozen storage and cultured to 70-80% confluence in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in an incubator maintained with 5% CO_2 at a temperature of 37˚C. The cells were then washed with PBS (Gibco; Thermo Fisher Scientific, Inc.) and digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.).

FaDu cell viability assay. The FaDu cells from the CON, NC and KD groups were seeded at a density of 5,000 cells/well in 96-well plates. Cell viability assays were performed at days 1, 3, 5 and 7 using an MTT assay (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Viable cells were quantified by measuring the absorption spectra at 490 nm with a Versamax microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The wells were observed daily using an Olympus CKX41 light microscope (Olympus Corporation, Tokyo, Japan). A total of 6 duplicate wells were used for each group. The samples were loaded into 96-well plates and the optical density values were measured at 490 nm with the spectrophotometer every 48 h. Growth curves were prepared using the mean values.

In vivo tumorigenicity assay of FaDu cells. A total of 20 non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (6 weeks of age; weight, 20-25 g) were obtained from Shanghai Si Laike Experimental Animals (Shanghai, China). NOD/SCID mice consisted of equal numbers of males and females. Food and water were available *ad libitum* and the mice were kept in standard laboratory conditions. Mice were maintained at a temperature of 23±2˚C, 65% humidity and 12-h light/dark cycles. Animals were randomly divided into three groups: A blank control group (CON, injected with FaDu cells infected
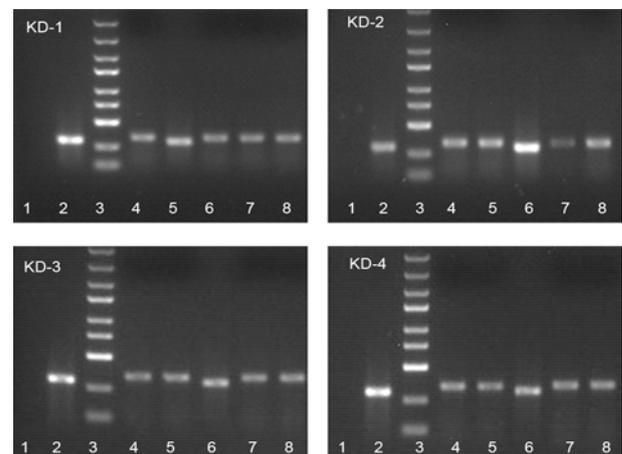
Figure 1. Analysis of recombinant cluster of differentiation 44 short hairpin RNA by polymerase chain reaction. Lane 1, negative control (double-distilled H_2O); lane 2, negative control (shRNA fragment not inserted, 307 bp); lane 3, markers from top to bottom: 5, 3, 2, 1.5, 1 kb, 750, 500, 250, 100 bp; lanes 4-8, 341-bp-long positive clones.

with PBS alone; n=8), a negative virus control group (NC, injected with FaDu cells infected with the negative control lentiviral vector; n=4) and an RNAi group (KD, injected with FaDu cells infected with the RNAi-CD44 lentiviral vector; n=8). Cells (1x10⁶) were suspended in 0.1 ml PBS and injected into the hypodermis of the right armpit of the NOD/SCID mice. Twice a week, the injection site of the NOD/SCID mice was palpated to check for tumor formation and growth. A total of 7 weeks after the injection (when the tumor size was ~1 cm in diameter), the mice were sacrificed using carbon dioxide euthanasia. The flow rate of CO_2 was 28% of the chamber volume/minute, and the volume of the cage was 294x190x130 mm. The final concentration of CO_2 was 80%. Euthanasia was confirmed through

Table III. Expression level of CD44 mRNA 5 days after target cell transfection with the low multiplicity of infection.

Groups	CD44/GAPDH	P-value[a]
NC	1.000±0.028	
KD1	0.300±0.019	8.4211×10^{-6}
KD2	0.261±0.008	2.1910×10^{-4}
KD3	0.430±0.047	2.6567×10^{-4}
KD4	0.472±0.025	1.5357×10^{-5}

[a]Comparison of NC and KD groups. CD44, cluster of differentiation 44; NC, negative control short hairpin RNA group; KD1, CD44-knockdown by shRNA 1.

Figure 3. Screening of the low multiplicity of infection group. CD44, cluster of differentiation 44; NC, negative control; KD1, CD44-knockdown by shRNA 1.

Table IV. Expression level of CD44 mRNA 5 days after target cell transfection with the high multiplicity of infection.

Groups	CD44/GAPDH	P-value[a]
NC	1.000±0.031	
KD1	0.236±0.010	1.9202×10^{-4}
KD2	0.168±0.001	4.6399×10^{-4}
KD3	0.493±0.034	4.7761×10^{-5}
KD4	0.539±0.020	9.5098×10^{-5}

[a]Comparison of NC and KD groups. CD44, cluster of differentiation 44; NC, negative control short hairpin RNA group; KD1, CD44-knockdown by shRNA 1.
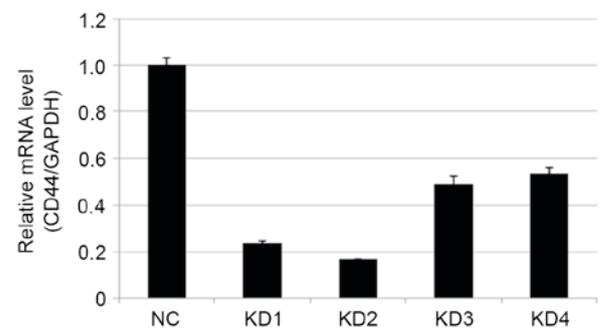
Figure 4. Screening of the high multiplicity of infection group. CD44, cluster of differentiation 44; NC, negative control; KD1, CD44-knockdown by shRNA 1.

Figure 2. Lentiviral transfection of FaDu cells. Green color denotes cells that have undergone successful green fluorescent protein-lentivirus co-transfection. CON, control (uninfected); NC, negative control shRNA virus; KD, CD44 shRNA lentivirus; bright, image captured using bright field microscopy; green, image captured using fluorescent microscopy. Magnification, x400.
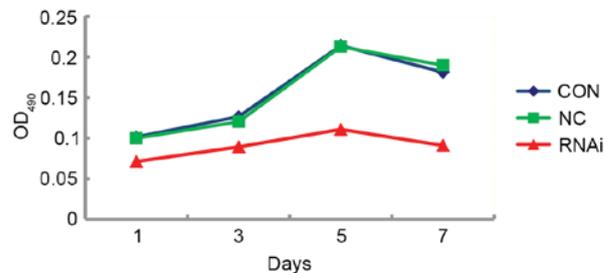
Figure 5. Growth curves of FaDu cells following treatment with cluster of differentiation 44 RNAi (or equivalent controls) *in vitro*. RNAi, RNA interference; CON, blank control group; NC, negative control.

the disappearance of physiological responses such as corneal reflexes, and the lack of detectable heart beat for 1 min. Tumor nodules were immediately extracted from the animals. The long axis (a) and short axis (b) of all tumors were measured and the tumor volume (V) was calculated as follows: $V = ab^2/2$. Finally, all tumors were histopathologically examined and diagnosed by two senior pathologists. The animal experiments were approved by the Animal Ethics Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China).

Statistical analysis. Data are expressed as the mean ± standard deviation, and were analyzed with SAS version 9.13 software (SAS Institute Inc., Cary, NC, USA). Statistical analyses were
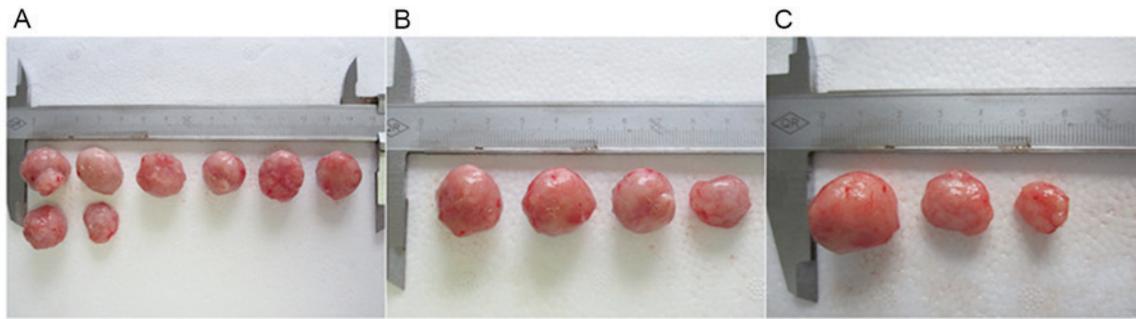
Figure 6. Tumorigenicity of FaDu cells following cluster of differentiation 44 gene-knockdown in mice. Transplantation assays of (A) the blank control group (tumorigenic rate, 8/8); (B) the negative virus control group (tumorigenic rate, 4/4); and (C) the RNA interference group (tumorigenic rate, 3/8).

performed on RT-qPCR results obtained through the $2^{-\Delta\Delta Cq}$ method. The distribution of tumor volumes was analyzed for normality using the Shapiro-Wilk test. The comparison of mean tumor volumes was conducted using one-way analysis of variance followed by LSD post-hoc test. Fisher's exact test was used to determine the difference in the number of tumorigenic mice between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Verification of recombinant lentiviral vectors targeting CD44. The four plasmids containing the four CD44-targeting shRNA clones, with the correct insertion and orientation of the shRNA fragment of the hU6-MCS-CMV-EGFP expression vector, were detected by PCR (Fig. 1). These recombinant lentiviral vectors (Lv-shRNA-CD44) were used to specifically knock-down CD44 expression.

Screening of lentiviral vectors targeting CD44. FaDu cells were transfected with Lv-shRNA-CD44 vectors (KD1, KD2, KD3 and KD4). Cells were examined by fluorescence micros-copy 72 h after lentivirus transduction (Fig. 2). On day 5 after transduction, cells were harvested to determine the efficiency of CD44 silencing by RT-qPCR (Figs. 3 and 4; Tables III and IV). Compared with the NC group, the most effective knock-down efficiency for the CD44 gene was >70%, using KD2 at a titer of 8E+8 TU/ml (P<0.01). Thus KD2, the most efficient recombinant vector, was used in subsequent experiments *in vitro* and *in vivo*.

Proliferative capabilities of FaDu cells following CD44 gene-knockdown in vitro. To evaluate the proliferative capa-bilities of FaDu cells *in vitro* following CD44 gene silencing, FaDu cells from the CON, NC and RNAi groups were seeded at the same density in 96-well plates, and the numbers of viable cells were quantified at days 1, 3, 5 and 7 using the MTT assay. This assay revealed distinct differences in cell growth curves *in vitro* between the RNAi group and the control groups. Although the growth rate was similar between CON and NC groups, the RNAi group had a significantly slower proliferation rate (P<0.05; Fig. 5).

Tumorigenicity of FaDu cells following CD44 gene knock-down in vivo. To assess the tumorigenic ability of FaDu cells

in vivo, 1×10^6 cells from each of the three groups were injected into the right armpit hypodermis of NOD/SCID mice. All mice were monitored for 7 weeks (Fig. 6; Table V). There was no significant difference in tumorigenesis between the CON and NC groups (Fisher's exact test, $\chi^2=1.726$, P>0.05), although there was a significant difference in tumorigenesis between the CON and RNAi groups (Fisher's exact test, $\chi^2=7.809$, P<0.05; Table VI). Mice from the RNAi group required a longer incubation period to develop tumors than mice from the CON group. In addition, the mean tumor volume in the CON group ($1,554.56\pm216.83$ mm^3) was not significantly different to the mean tumor volume in the NC group ($1,512.38\pm239.86$ mm^3) (t=0.31, P>0.05), while the mean tumor volume in the RNAi group ($1,061.00\pm185.76$ mm^3) was significantly smaller than the CON group (t=3.47, P<0.05) (Table VII). The tumorigenic ability of FaDu hypopharyngeal cancer cells was therefore significantly weaker following knockdown of the CD44 gene. These observations suggest that the CD44 gene may serve an important role in the cancer stem cell properties of CD44+ cells in the FaDu hypopharyngeal cancer cell line. A pathological study confirmed that all tumors were poorly differentiated squamous cell carcinomas with cellular hetero-geneity (Fig. 7).

Discussion

Recent data have indicated that tumor stem cells exist in numerous types of malignant tumors (2-10), including head and neck malignant tumors (11-14), and are closely associated with the occurrence, development and metastasis of malignant tumors (14,18-21). Hypopharyngeal carcinoma is a head and neck malignant tumor with one of the poorest prognoses, and the mechanisms of its tumor development and progression are unclear (22). Results from a previous study demonstrated that CD44+ cells in hypopharyngeal cancer had a stronger prolif-erative capability than CD44- cells and a higher tumorigenic potential, indicating that cancer stem cells in hypopharyngeal cancer may exist in the CD44+ tumor cell population, or that CD44+ cells may be hypopharyngeal cancer stem cells (15). To investigate whether the CD44 gene confers the biological characteristics of CD44+ cancer stem cells in hypopharyn-geal cancer, the tumorigenicity of CD44+ cells required the knockout or knockdown of the CD44 gene. Previous studies have demonstrated that RNAi is an economical, fast and highly efficient technique for knocking down gene expression (23-26).

Table V. Tumorigenic rate of FaDu cells *in vivo* following CD44 RNAi.

Groups	Tumor incidence (incubation period, days)	Tumorigenicity rate, %
CON	8/8[a] (14, 13, 16, 17, 14, 16, 19, 18)	100
RNAi	3/8 (27, 24, 25)	37.5[b]
NC	4/4 (16, 17, 19, 14)	100

[a]Number of tumors formed/number of effective injections. [b]The tumorigenic rate of the RNAi group was significantly lower than the CON and NC groups (Fisher's exact test, χ^2=7.809, P<0.05). CON, control group; RNAi, RNA interference; NC, negative control short hairpin RNA group.

Table VI. Number of nude mice that formed or failed to form tumors.

Groups	No. of mice with tumor failure (n=5)	No. of mice with tumor formation (n=15)
Control	0	8
RNAi-CD44	5	3
NC-RNAi	0	4

CD44, cluster of differentiation 44; NC, negative control.

Table VII. Tumor volumes in mice injected with FaDu cells following transfection with CD44 RNAi.

Groups	Long axis, mm	Short axis, mm	Tumor volume, mm³	Mean tumor volume, mm³
CON	17	15	1912.5	1554.56±216.83
	15	13	1267.5	
	16	14	1568.0	
	15	14	1470.0	
	17	13	1436.5	
	17	14	1666.0	
	18	14	1764.0	
	16	13	1352.0	
RNAi	14	12	1008.0	1061.00±185.76[a]
	15	13	1267.5	
	15	11	907.5	
NC	18	14	1764.0	1512.38±239.86[b]
	17	14	1666.0	
	16	13	1352.0	
	15	13	1267.5	

[a]Difference in the mean volumes was statistically significant between the RNAi and CON groups (t=3.47, P<0.05). [b]The difference in the mean volumes was not statistically significant between the NC and CON groups (t=0.31, P>0.05). CON, control group; RNAi, RNA interference; NC, negative control short hairpin RNA group.
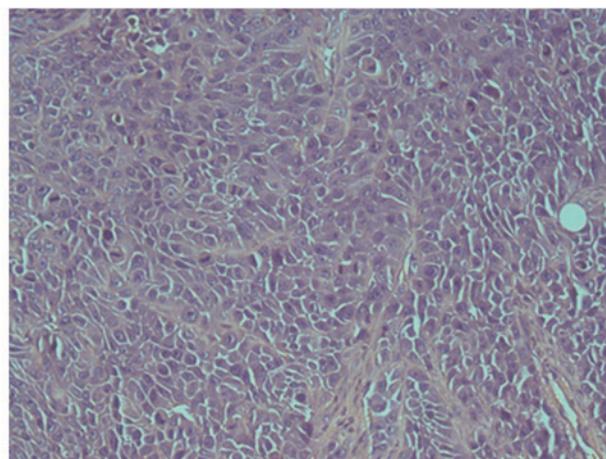
Figure 7. Tumorigenic experiment *in vivo*. A representative image from the tumor of mice injected with FaDu cells subcutaneously. Pathological assessment by hematoxylin and eosin staining confirmed that all the tumors were poorly-differentiated squamous cell carcinoma. Magnification, x200.

Thus, RNAi technology was used to suppress the expression of CD44 in the present study.

To confirm the role of CD44 in cancer stem cells in hypopharyngeal cancer further, RNAi technology was used to knockdown CD44 gene expression in FaDu hypopharyngeal cancer cells and any changes in their tumorigenicity were assessed. Following RNAi of the CD44 gene, the expression level of CD44 mRNA was significantly decreased, with a silencing efficiency >70% (reflective of successful gene silencing). The proliferative capacities of FaDu cells *in vitro* following CD44-silencing differed between the KD group and the CON or NC groups, which indicated that FaDu cells have a slower growth rate following CD44 gene-knockdown. Subsequent to the injection of FaDu cells into NOD/SCID mice, the tumorigenic rate in the KD group was significantly lower than the CON or NC group. Mice from the KD group also required a longer incubation period to develop tumors compared with mice in the CON or NC group. Furthermore, the tumor volumes in the KD group were markedly smaller than those in the CON or NC groups. In conclusion, the tumorigenic potential of FaDu cells became significantly weaker once CD44 expression was knocked down by RNAi. These observations suggest that the CD44 gene may play an important role in the cancer stem cell properties of CD44+ cells in hypopharyngeal cancer.

The results from the present study and a previous study (15) indicate that CD44 is an important biological marker of hypopharyngeal cancer stem cells. The results of the present study also indicate that FaDu cells retained proliferative abilities *in vitro* and tumorigenicity *in vivo* following CD44 gene-knockdown; thus, it cannot be considered that CD44 is a completely unique molecular biomarker for hypopharyngeal cancer or that all biological characteristics of stem cells in hypopharyngeal cancers are conferred by the CD44 gene. The results of the current study continue to suggest that CD44+ cells are notable cancer stem cells in hypopharyngeal cancer, and that certain biological characteristics of cancer stem cells in hypopharyngeal cancer are conferred by the CD44 gene.

Other molecular markers of hypopharyngeal cancer stem cells, and the precise role of CD44 amongst all molecular markers of hypopharyngeal cancer stem cells, require further study.

Acknowledgements

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