Tumor suppressor function of *BCSC-1* in nasopharyngeal carcinoma

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BCSC-1 is dramatically upregulated in CNE-2L2 human nasopharyngeal carcinoma cells with reduced malignancy (AS cells) and is proposed to be a candidate tumor suppressor gene. We therefore examined the effect of *BCSC-1* expression on malignant behaviors of CNE-2L2 cells. Growth *in vitro* and tumorigenesis in nude mice of wild-type CNE-2L2 cells (W cells) were inhibited by ectopic *BCSC-1*, and those of AS cells were promoted by *BCSC-1* suppression. The tumor suppressor function of *BCSC-1* was further confirmed by a study showing that intratumor *BCSC-1* injection caused growth suppression of the tumor from W cells inoculated in nude mice. Immunohistochemistry exhibited marked reduction of *BCSC-1* expression in 11 of 39 human nasopharyngeal carcinoma specimens. Because *BCSC-1* expression was as rich as that in normal cells in the rest of the carcinoma specimens and was poor in CNE-2L2 cells, HNE-1 human nasopharyngeal carcinoma cells with rich *BCSC-1* expression were used as a control in the study. No effect of *BCSC-1* transfection on growth of the cells was observed. The data suggest that *BCSC-1* suppression might play roles in tumorigenesis of some nasopharyngeal carcinomas and that *BCSC-1* might be a potential gene therapy target in nasopharyngeal carcinomas with poor *BCSC-1* expression. Enhanced aggregation of cells together with increased E-cadherin and α-catenin expression and reduced Wnt signaling might be involved in the mechanisms of tumor suppressor function of *BCSC-1*. (*Cancer Sci* 2009; 100: 1817–1822)

We previously observed that α-mannosidase *Man2c1* suppression causes profound inhibition of malignant activities of the human nasopharyngeal carcinoma-derived cell line CNE-2L2.[1] In order to explore possible mechanisms of malignancy inhibition, mRNA differential display analysis was carried out on CNE-2L2 cells with *Man2c1* suppression (called AS cells) and CNE-2L2 cells transfected with mock (called M cells). Of the 1069 genes examined, 28 were upregulated and 31 were downregulated in AS cells. LOH11CR2A, also called *BCSC-1* (GenBank no. NM_014622), was identified as the most dramatically upregulated gene. *BCSC-1* cDNA was originally cloned and proposed to be a candidate tumor suppressor gene (TSG) by Martin *et al.*[2] Therefore, association of *BCSC-1* with malignant behaviors of CNE-2L2 cells was examined in this study.

It was observed that growth and tumorigenesis of wild-type CNE-2L2 cells (W cells) were inhibited by ectopic *BCSC-1* whereas those of AS cells were promoted by *BCSC-1* suppression, indicating that *BCSC-1* is a TSG in CNE-2L2 cells. The tumor suppressor function was further confirmed by a study showing that intratumor *BCSC-1* injection results in growth inhibition of tumors from W cells inoculated in nude mice. *BCSC-1* expression was examined by means of immunohistochemistry in human nasopharyngeal carcinomas. Because only some carcinoma specimens showed marked reduction of *BCSC-1* expression and *BCSC-1* expression was poor in CNE-2L2 cells, the HNE-1 nasopharyngeal carcinoma cell line with rich

BCSC-1 expression was used as a control in the present study. The data obtained suggest that *BCSC-1* suppression might play roles in tumorigenesis of some nasopharyngeal carcinomas and *BCSC-1* might be a potential target in gene therapy of those nasopharyngeal carcinomas with poor *BCSC-1* expression.

TSG have been reported to exhibit multiple inhibitive effects on tumorigenesis.[3] Tumorigenesis, including that of nasopharyngeal carcinoma,[4] is a very complicated process and is regulated by a number of factors.[5,6] Therefore, it could be postulated that the suppressive effect of *BCSC-1* on malignant behaviors of CNE-2L2 cells would be at multiple points. Because reduced cell adhesion caused by decreased E-cadherin expression[7] and activation of the Wnt signaling pathway[8] were found in nasopharyngeal carcinoma, the effect of *BCSC-1* expression on cell aggregation, E-cadherin expression, and Wnt signaling was examined. It was observed that ectopic *BCSC-1* resulted in enhanced cell aggregation associated with increased expression of E-cadherin/α-catenin, and *BCSC-1* suppression promoted Wnt signaling.

Materials and Methods

Cell lines, tissue sections, and animals. The CNE-2L2 human nasopharyngeal carcinoma cell line was developed by Yu and Gao.[9] The HNE-1 human nasopharyngeal carcinoma cell line was developed by Yao *et al.*[10] CNE-2L2 cells with *Man2c1* suppression (AS cells) and CNE-2L2 cells with mock transfection (M cells) were developed by Yue *et al.*[1] GP-293 and Hek293 cells were obtained from the Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Culture medium RPMI-1640, DMEM, and FCS were purchased from Gibco-BRL (Frederick, MD, USA). Biopsy specimen sections (5 µm) of human nasopharyngeal tissue and nasopharyngeal carcinoma were provided by the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). Female 8-week-old BALB/c mice and female 8-week-old BALB/c (nu/nu) mice were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China).

Microarray analysis. mRNA differential display analysis on a BioStarH-I DNA chip (oligonucleotide array for 1069 human genes; BioStar Genechip, Shanghai, China) was carried out to examine the effect of *Man2c1* suppression on gene expression of CNE-2L2 cells (Supporting information: Materials and methods).

Development of the anti-BCSC-1 protein mAb. A BCSC-1 fusion protein expressed by pET-30a-*BCSC-1* in *Escherichia coli* was purified and used for immunization of mice and screening of

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hybridomas (Supporting information: Materials and Methods). Immunization of 8-week-old BALB/c mice, cell fusion, and hybridoma selection were conducted according to procedures described previously.[11] Isotype detection was carried out using Isostrip Monoclonal Antibody Isotyping Kit (Roche Shanghai, Shanghai, China). Ascites were produced in BALB/c mice and mAb purification from ascites was carried out using ammonium sulfate and then caprylic acid following procedures described previously.[11]

ELISA. ELISA was carried out on the BCSC-1 fusion protein. April fusion protein expressed by pET-30a-April (kindly provided by Ms Ling Zhang, Department of Immunology, Peking Union Medical College, Beijing, China) was used as a negative control.

Western blot analysis. Western blotting was carried out following procedures described previously.[1] The mAb anti-BCSC-1, mAb anti-hE-Cadherin (clone SHE78-7; Zymed, South San Francisco, CA, USA), and mAb anti-hα-catenin or hβ-catenin (Santa Cruz, Santa Cruz, CA, USA) were used as the primary antibodies. Goat anti-mouse IgG conjugated with horseradish peroxidase (BD Biosciences, San Jose, CA, USA) was used as the secondary antibody. β-Actin was used as an internal control. The protein bands were visualized using SuperSignal West Pico Trial Kit (Pierce, Rockford, IL, USA).

Stable transfection of cells with BCSC-1. The PCR-amplified product from pUCm-T-*BCSC-1* using BCSC upstream 1 and downstream 1 primers (Supporting information: Table 1) was inserted into *Eco*RI- and *Xba*I-digested pcDNA4/*myc*-His A (Invitrogen, Carlsbad, CA, USA). pcDNA4/*myc*-His A or pcDNA4/*myc*-His A-*BCSC-1* was transfected into W cells upon the action of Lipofectamine (Gibco-BRL). Zeocin-resistant cell pools (BCSC-1 and mock cells) were obtained after 7 days of culture in medium containing zeocin (50 μg/mL).

Suppression of BCSC-1 by siRNA. Four siRNA oligonucleotides specific to *BCSC-1* (Supporting information: Table 1) were used in the present study. siRNA was annealed and inserted into *Mlu*I- and *Xho*I-digested pRNA-H1.1/Retro (Genscript, Piscataway, NJ, USA). A control plasmid generating shRNA with non-silencing RNA was also constructed. Generation of recombinant retrovirus was carried out according to the procedures introduced by Liu *et al.*[12] When GP-293 cell culture reached 70–80% confluence in six-well plates, recombinant plasmids and pVSVG mixed with Lipofectamine were applied to the cell culture according to the manufacturer's instructions. The recombinant retrovirus produced was used to infect AS cells. After 12 h, the culture medium was replaced with fresh medium supplemented with hygromycine (400 μg/mL) (Sigma, St Louis, MO, USA). After 7–10 days, hygromycine-resistant cell pools called si-1, si-2, si-3, si-4, and Retro infected with the virus harboring the control plasmid were developed.

RT-PCR and real-time PCR. Total RNA was extracted from cells with Trizol reagent (Gibco-BRL). cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA). PCR was carried out using 2 μL of cDNA in a 50-μL reaction system at 94°C for 5 min, then 94°C for 45 s, 58°C for 45 s, and 72°C for 90 s for 30 cycles, followed by 72°C for 5 min for the final extension. PCR products were examined by electrophoresis on a 1% agarose gel containing ethidium bromide. Real-time PCR was run using the SYBR Premix Ex *Taq* Kit (Takara Beijing, Beijing, China) according to the instructions, using the BCSC upstream 2 and downstream 2 primers (Supporting information: Table 1). PCR microplate was the product of Axygen (Union City, CA, USA). The data for *BCSC-1* was standardized to *β-actin*. *BCSC-1* mRNA/*β-actin* mRNA for W cell (Fig. 1a, Fig. 2a left panel) and for AS cell (Fig. 2a right panel) was normalized as 1.

Cell proliferation assay. Cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega). Five hundred cells per well in 200 μL DMEM (Gibco-BRL) containing 10% FCS were seeded into 96-well
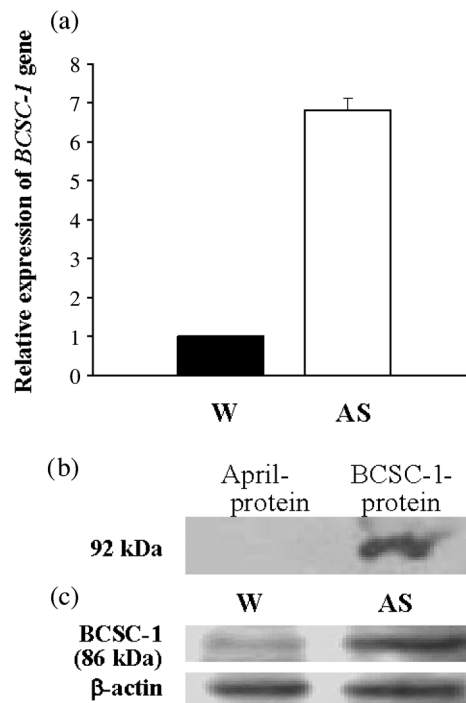
Fig. 1. Upregulation of *BCSC-1* in AS cells. (a) Real-time PCR using the SYBR Premix Ex *Taq* Kit. The data for *BCSC-1* was standardized to β-actin. For W cells the level of β-actin mRNA was set at 1. (b,c) Western blotting carried out on recombinant proteins (b) and proteins extracted from W and AS cells (c) with mAb anti-BCSC-1 as a probe. The deduced molecular weight of the BCSC-1 protein is 86 kDa. W and AS represent wild-type CNE-2L2 cells and CNE-2L2 cells with *Man2c1* suppression respectively.

plates (Corning, Coening, NY, USA). On days 1, 2, 3, 4, 5, and 6, 20 μL of assay solution was applied to each well and the plates were incubated at 5% CO_2, 37°C for 2.5 h. The absorbance was measured at 490 nm. The assays were carried out three times.

Colony formation. Colony formation was carried out in soft agar according to procedures described previously.[1] Suspensions containing 1×10^4 cells were seeded into the top of the agar. After 14 days incubation at 5% CO_2, 37°C, the number of colonies with a diameter >500 μm was counted and the colony formation rate was calculated. Counts were expressed as the number of colonies per plate on average from three independent experiments.

Cell aggregation assay. The cell aggregation assay was carried out according to procedures described previously.[1] Cell aggregation was examined under a reverse microscope.

Tumorigenicity in nude mice. Cells (1×10^6) in DMEM without serum (0.2 mL) were subcutaneously inoculated into the right armpit of nude mice with seven mice in each group. The longest (*a*) and shortest (*b*) diameters of tumors were measured weekly for 8 weeks. Tumor volume was calculated according to the formula: volume $= 4/3\pi a \times b^2$. Tumors were excised under anesthesia and weighed at the end of week 8.

Construction of recombinant adenovirus vector and preparation of adenovirus stocks. *Eco*RI- and *Sal*I-excised *BCSC-1* from pET-30a-BCSC-1 was inserted into the shuttle plasmid pDC316 (Microbix Biosystems, Toronto, Canada). Adenovirus (Ad5-BCSC-1) was developed by the Gene Technology Company (Beijing, China). Briefly, pDC316-*BCSC-1* was cotransfected with the genomic plasmid pBHGlox_E1, 3Cre (Microbix Biosystems) into Hek293 cells by calcium phosphate precipitation. The adenovirus (Ad5-BCSC-1) was purified by means of cesium chloride gradient ultracentrifugation. Viral titer was determined

as infectious units (IFU) using TCIG50 end-point dilution. The final titer of the purified viral vectors was 5.7×10^9 IFU/mL. The virus stocks were aliquoted and stored at –80°C. Ad5-egfp was purchased from the Gene Technology Company.

Adenovirus-mediated gene therapy in nude mice. Each BALB/c mouse was subcutaneously inoculated with 2×10^6 W cells at the left armpit. The experiment was divided into three groups with six mice in each group. When tumors reached the size of 50–100 mm^3, 0.1 mL PBS, 0.1 mL Ad5-egfp (4×10^8 IFU), or 0.1 mL Ad5-BCSC-1 (4×10^8 IFU) was injected into the tumor in each mouse for groups 1, 2, or 3 respectively. The injection was repeated 1 week later. A further 2 weeks later, tumors were excised under anesthesia, size-measured, and weighed. Tumor volume was calculated according to the formula mentioned above.

Immunohistochemistry. Immunohistochemistry was carried out using the Biotin SP-HRP (DAB) Kit (Tianjin Bomeike Biotech, Tianjin, China) following the manufacturer's protocol. The mAb anti-BCSC-1 was used as the primary antibody.

Multiple protein sequence alignments. The homologous proteins were searched from the NCBI database using the HomoloGene program. Multiple protein sequence alignments were carried out using the CLUSTALW program.

Results

BCSC-1 protein is highly conserved during evolution. Homologene presents nine homologues of BCSC-1 in *Homo sapiens* (NM_014622), *Pan troglodytes* (XP_001137734.1), *Canis lupus familiaris* (XP_546453.2), *Bos Taurus* (XP_600048.3), *Mus musculus* (NP_766355.2), *Rattus norvegicus* (NP_942050.1), and *Danio rerio* (XP_691937.3, XP_001336637.2 and XP_001920968.1). These proteins exhibit high homology of sequence (Supporting information Fig. 1), indicating that BCSC-1 is a highly conserved protein in evolution.

Upregulation of *BCSC-1* in CNE-2L2 cells with *Man2c1* suppression. To explore possible mechanisms of reduced malignancy of CNE-2L2 cells by *Man2c1* suppression, mRNA differential display analysis between cells with *Man2c1* suppression (AS cells) and cells transfected with mock (M cells) was carried out. Of the 1069 genes examined, 28 were upregulated and 31 were downregulated in AS cells. LOH11CR2A, also called *BCSC-1* (GenBank no. NM_014622), was identified as the most dramatically upregulated gene (Supporting information: Table 2). Real-time PCR validated the result obtained from microarray analysis (Fig. 1a).

In order to further examine *BCSC-1* expression in cells, the mAb anti-BCSC-1 was developed. BCSC-1 and April fusion proteins were used for screening of hybridoma supernatants. The supernatants positive for BCSC-1 but negative for April in ELISA were rescreened by western blotting. Figure 1(b) shows a supernatant reactive to BCSC-1 fusion protein but not to April fusion protein (the deduced molecular weight of BCSC-1 protein is 86 kDa). Western blotting in Figure 1(c) shows a mAb with much stronger reactivity to the ~86 kDa band in AS cells compared with W cells, further confirming enhanced *BCSC-1* expression in AS cells and indicating that the mAb also recognizes natural BCSC-1. The mAb was used in immunohistochemistry. Supporting information: Figure 2 shows that the staining with the mAb on the tumor from AS cells was much stronger than on the tumor from W cells inoculated in nude mice.

Suppressive effect of *BCSC-1* on growth of CNE-2L2 cells with poor *BCSC-1* expression but not on HNE-1 cells with rich BCSC-1 expression. The human nasopharyngeal carcinoma-derived cell lines CNE-2L2 (with poor *BCSC-1* expression) and HNE-1 (with rich *BCSC-1* expression) were used in the present study. BCSC-1-C1 and BCSC-1-C2 were two cell pools from wild-type CNE-2L2 cells (W cells) transfected with *BCSC-1*.

BCSC-1-H was a cell pool from HNE-1 cells transfected with *BCSC-1*. Mock-C and mock-H were the cell pools from W cells and HNE-1 cells transfected with mock respectively. *BCSC-1* expression was markedly enhanced in BCSC-1-C1 and BCSC-1-C2 cells (Fig. 2a,b, left panel), but not in BCSC-1-H cells (Fig. 2e, left panel). Growth in culture (Fig. 2c, left panel) and colony formation (Fig. 2d, left panel) of BCSC-1-C1 and BCSC-1-C2 cells were markedly reduced. However, no effect of *BCSC-1* transfection on HNE-1 cell growth was observed (Fig. 2e, right panel). si2 and si4 were two cell pools infected with the virus harboring siBCSC-1-2 and siBCSC-1-4, respectively, and showed profound suppression of *BCSC-1* expression (Fig. 2a,b, the right panels). *BCSC-1* suppression promoted cell growth in culture (Fig. 2c, the right panel) and colony formation (Fig. 2d, the right panel) of AS cells, further confirming the suppressor function of *BCSC-1* on CNE-2L2 cell growth.

Tumorigenesis suppression of CNE-2L2 cells by *BCSC-1*. Tumor growth curves showed that the tumors from BCSC-1-C1 or BCSC-1-C2 cells inoculated in nude mice grew much slower than those from controls (Fig. 3a). The average weights of the tumors at the end of the study were 4.05 ± 2.70, 3.07 ± 1.90, 0.65 ± 0.28, and 1.10 ± 0.70 g for W cells, M cells, BCSC-1-C1 cells, and BCSC-1-C2 cells, respectively. The difference between W or M and BCSC-1-C1 or BCSC-1-C2 was statistically significant (paired *t*-test, $P < 0.01$). In contrast, *BCSC-1* suppression promoted tumorigenesis of AS cells (Fig. 3b). All but one of the mice inoculated with si-2 cells and four mice inoculated with si-4 cells died of tumor growth in the middle of the experiment. The tumor in the mouse inoculated with si-2 cells remaining alive was 60.9 cm^3 in size and 4.03 g in weight. The average weights of the tumors at the end of the study were 2.52 ± 0.23, 2.28 ± 0.42, and 4.79 ± 1.27 g for AS cells, Retro cells, and si-4 cells respectively. The difference between AS or Retro and si-4 was statistically significant (paired *t*-test, $P < 0.01$).

To further confirm the tumor suppressor function of *BCSC-1*, experimental tumor gene therapy with *BCSC-1* was carried out. When tumors from inoculated W cells in nude mice reached the size of 50–100 mm^3, Ad5-*BCSC-1*, Ad5-egfp, or PBS was injected into tumors. Two weeks after the second virus injection, the average tumor size of the PBS group, Ad5-egfp group, and Ad5-*BCSC-1* group was 3.14 ± 0.85, 3.61 ± 0.89, and 0.80 ± 0.41 cm^3 respectively. The difference between PBS or Ad5-egfp and Ad5-*BCSC-1* was significant (paired *t*-test, $P < 0.01$). A tumor size comparison is shown in Figure 4.

Effect of BCSC-1 on cell aggregation, E-cadherin/α-catenin/β-catenin expression, and Wnt signaling. Ectopic *BCSC-1* promoted aggregation of CNE-2L2 cells (Fig. 5a) and E-cadherin and α-catenin expression (Fig. 5b). *BCSC-1* suppression resulted in upregulation of Wnt3, Wnt4, Wnt5a, Wnt6, Wnt10b, Fzd4, and Tcf3 (Fig. 5c) and enhanced expression of β-catenin (Fig. 5d). Expression of the remainder of the Wnt signaling pathway was not affected by *BCSC-1* suppression. Expression of Wnt family members was assayed by RT-PCR using the primers listed in Supporting information: Table 1.

BCSC-1 expression in human nasopharyngeal carcinomas. Immunohistochemistry was carried out to examine *BCSC-1* expression in three human normal nasopharyngeal tissue specimens and 39 human nasopharyngeal carcinoma specimens. Normal nasopharyngeal epithelial cells in the three specimens showed rich *BCSC-1* expression. Some lymphoid cells in the interstitial tissues also showed rich *BCSC-1* expression. Eleven of 39 carcinoma specimens (28.2%) exhibited marked reduction of *BCSC-1* expression whereas *BCSC-1* expression in the rest was as rich as that in normal cells. Supporting information: Figure 3 shows examples of *BCSC-1* expression in normal nasopharyngeal tissues and in nasopharyngeal carcinomas assayed by immunohistochemistry.
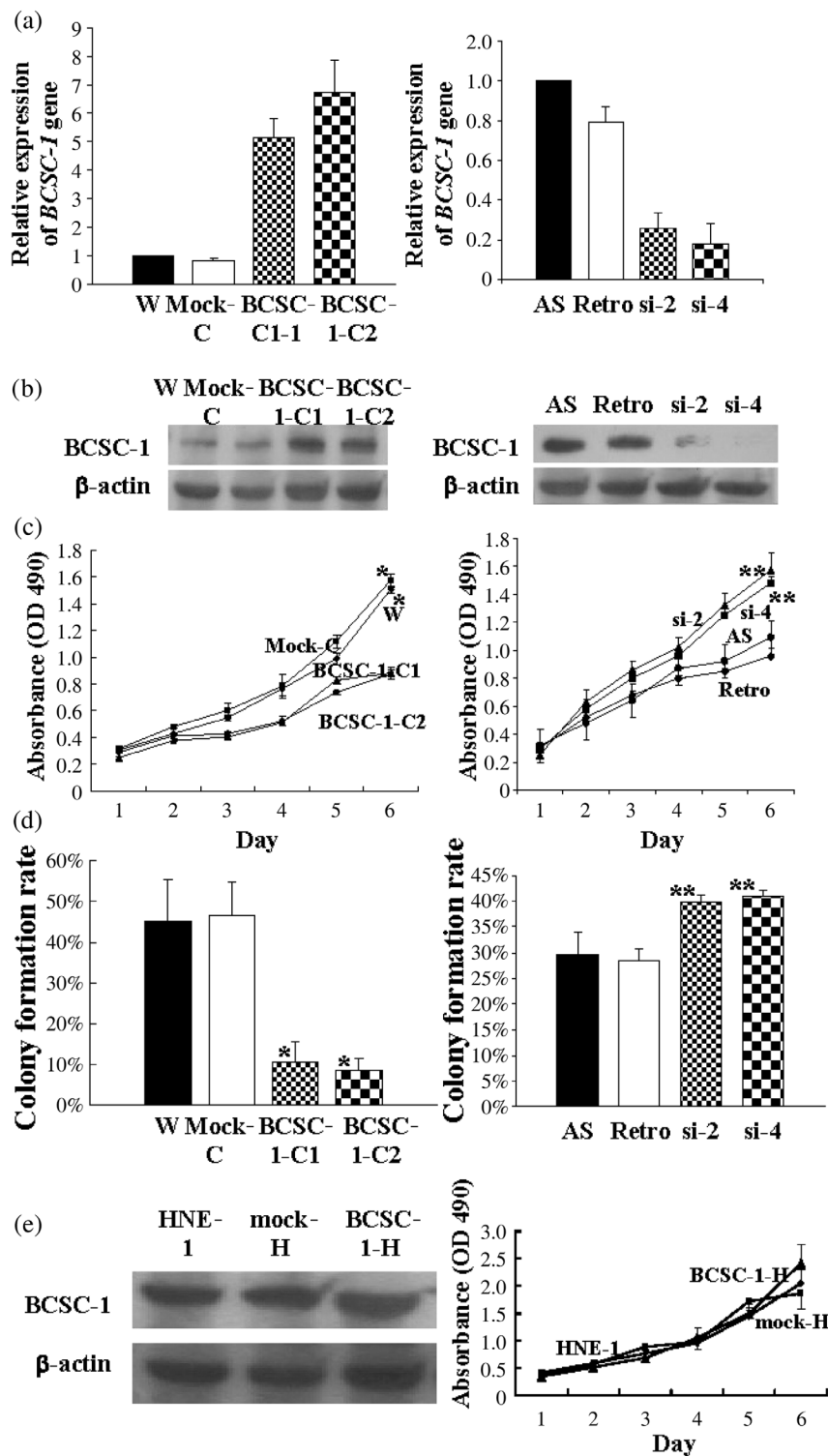
Fig. 2. Suppressive effect of *BCSC-1* on the growth of CNE-2L2 cells but not on that of HNE-1 cells. *BCSC-1* expression was examined by (a) real-time PCR and (b) western blotting with the designated mAb anti-BCSC-1 protein as a probe (b, the left panel of e). For cell growth test, 500 cells per well were seeded into 96-well plates. (c, the right panel of e) Cell growth curves. Colony formation assay was carried out in soft agar. (d) Colony formation rate. The data are expressed as the means of three experiments. W, mock-C, mock-H, and Retro represent wild-type CNE-2L2 cells, CNE-2L2 cells transfected with pcDNA4/*myc*-His A, HNE-1 cells transfected with pcDNA4/*myc*-His A, and CNE-2L2 cells infected with retrovirus harboring a control plasmid, respectively. BCSC-1-1 and BCSC-1-2 are two W cell pools with ectopic *BCSC-1*. si-2 and si-4 are two AS cell pools with *BCSC-1* suppression. HNE-BCSC-1 is HNE-1 cells transfected with *BCSC-1*. *The difference between W or mock and BCSC-1-1 or BCSC-1-2 is statistically significant (paired *t*-test, $P < 0.01$). **The difference between AS or Retro and si-2 or si-4 was statistically significant (paired *t*-test, $P < 0.05$).

Discussion

BCSC-1 (NM_014622) is a newly characterized candidate TSG located at chromosome 11q23 encoding a 786-amino acid protein with a predicted molecular mass of 86 kDa.[2] Sequence alignment of nine BCSC-1 protein homologues shows high homology of the sequences, indicating that BCSC-1 is a highly conserved protein in evolution. Based on the proposal by Martin *et al.* that *BCSC-1* is a candidate TSG[2] and the microarray data

showing enhanced *BCSC-1* expression in the CNE-2L2 cells with reduced malignancy caused by α-mannosidase *Man2c1* suppression, the effect of *BCSC-1* expression on malignant behaviors of human nasopharyngeal carcinoma cells was examined in the present study. *BCSC-1* was found to be a TSG in CNE-2L2 cells.

Tumorigenesis is a very complicated process and is regulated by a number of factors.[4–6] TSG have been found to exhibit multiple inhibitive effects on tumorigenesis.[3] For example, the
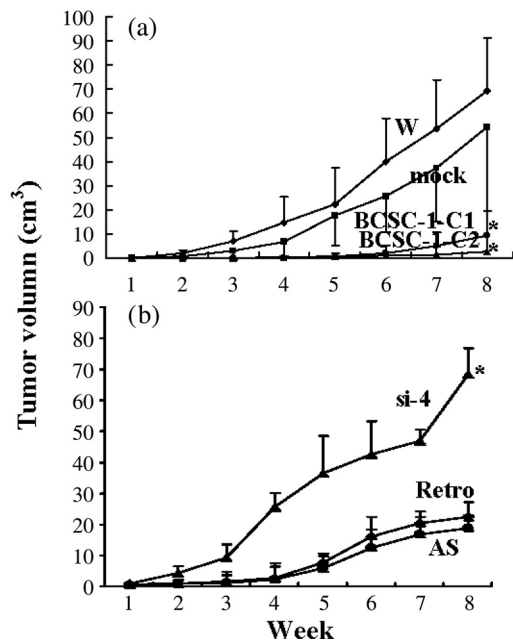
Fig. 3. Suppression of tumorigenesis of CNE-2L2 cells by *BCSC-1*. A total of 5×10^5 cells were subcutaneously inoculated into the right armpit of nude mice with seven mice in each group. The longest (*a*) and shortest (*b*) diameters of the tumors were measured weekly for 8 weeks. The volume of the tumors was calculated according to the formula: volume = $4/3\pi a \times b^2$. (a) (b) Growth curves of tumors. *The difference between wild type (W) or Mock and BCSC-1-1 or BCSC-1-2 and between AS or Retro and si-4 was statistically significant (paired *t*-test, $P < 0.01$).
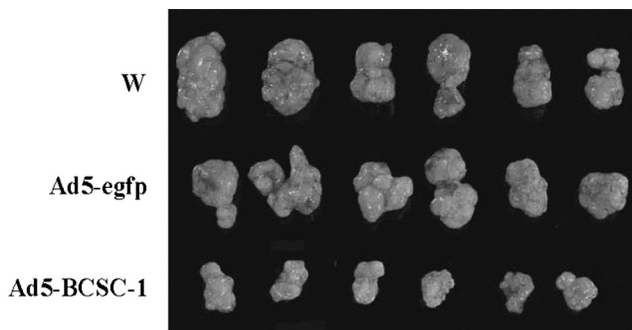
Fig. 4. Growth suppression of CNE-2L2 tumors by intratumor *BCSC-1* injection. A suspension containing 1×10^6 wild-type CNE-2L2 cells (W) was subcutaneously inoculated into each nude mouse. When tumors grew to 50–100 mm³, tumors in each mouse of groups 1, 2, and 3 were injected with 0.1 mL PBS, 0.1 mL Ad5-egfp (4×10^8 infectious units [IFU]) or 0.1 mL Ad5-BCSC-1 (4×10^8 IFU), respectively. The injection was repeated 1 week later. Two weeks after the second injection, tumors were excised. The figure shows a size comparison of the tumors.

retinoblastoma (RB) gene was reported to both govern the passage of cells through the G_1 phase-restriction point[13] and promote terminal differentiation.[14] In addition, Rb has also been observed to play roles in ensuring proper chromosome functions such as mitotic progression, faithful chromosome segregation,[15] checkpoint control,[16] and chromatin remodeling,[17] suggesting that Rb is involved in the maintenance of genome integrity. Tumorigenesis of nasopharyngeal carcinoma is also a very complicated process and is associated with a number of factors.[4] Because activation of the Wnt signaling pathway[8] and reduced cell adhesion caused by decreased E-cadherin[7] were reported in nasopharyngeal carcinoma, the effect of *BCSC-1* expression on Wnt signaling, E-cadherin expression, and aggregation of CNE-2L2 cells were examined. It was observed that
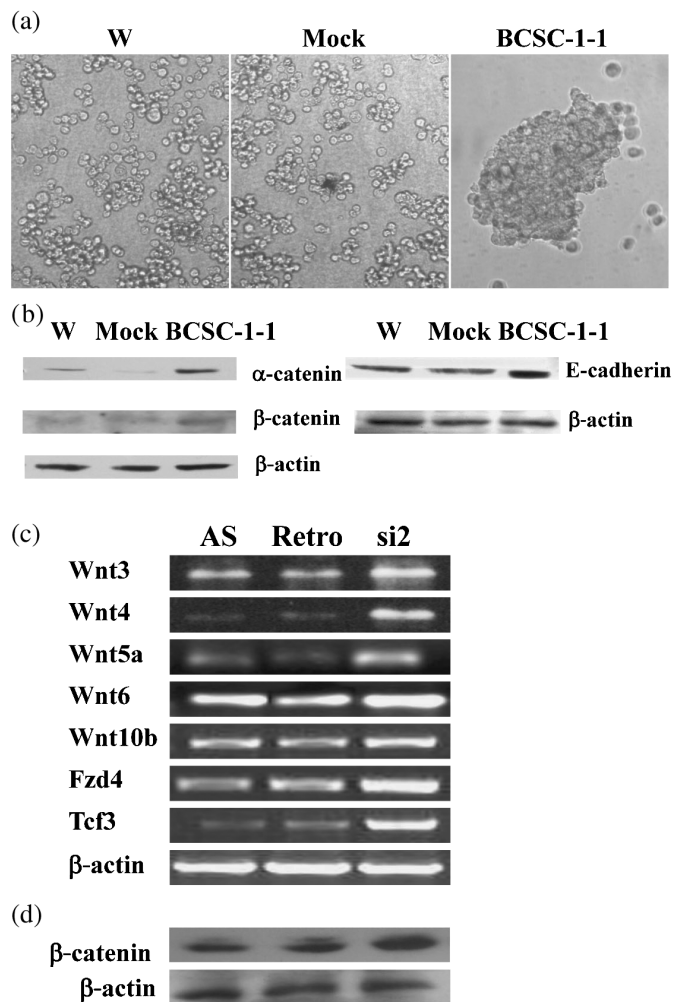
Fig. 5. Effect of *BCSC-1* expression on cell aggregation, expression of E-cadherin and α-catenin and β-catenin, and Wnt family members. A total of 1×10^5 cells were suspended in 1 mL HBSS-Ca²⁺ and incubated for 60 min at 5%CO₂, 37°C with intermittent gentle shaking. (a) Cell aggregation was examined using a reverse microscope. (b,d) Western blot analysis was carried out with mAb anti-E-cadherin, anti-α-catenin, or anti-β-catenin as the primary antibody. RT-PCR for Wnt family members was carried out with the primers listed in Supporting information: Table 1C. (a) and (b) shows that ectopic *BCSC-1* results in enhanced agression of and increased E-cadherin. (c) and (d) show that BCSC-1 suppression causes enhanced Wnt-signaling and β-cat enin in AS cell respectively.

ectopic *BCSC-1* caused enhanced aggregation of cells together with increased expression of E-cadherin/α-catenin. In epithelial cells, cell adhesion is mainly mediated by E-cadherin,[18] which functions through complex formation with catenins.[19] Loss or reduction of E-cadherin expression was reported to be correlated with reduced aggregation and dedifferentiation of many carcinomas.[20,21] Therefore, enhanced aggregation of cells associated with increased expression of E-cadherin/α-catenin would be one of the mechanisms associated with the suppressive effect of *BCSC-1* on the malignant behaviors of CNE-2L2 cells. The Wnt pathway is one of the cell proliferation signaling pathways, enhancement of which plays important roles in tumorigenesis.[22,23] The Wnt pathway is either β-catenin-dependent or β-catenin-independent.[23] In the present study expression of all members of the Wnt family together with their cell surface receptors was assayed by RT-PCR and that of β-catenin was examined by western blotting in whole cells. Upregulation of Wnt3, Wnt4,

Wnt5a, Wnt6, Wnt10b, Fzd4, and Tcf3 together with β-catenin in si-2 cells suggests that *BCSC-1* suppression would enhance Wnt signaling, which might play roles in the malignancy recovery of the cells.

With regard to possible mechanisms associated with enhanced *BCSC-1* expression induced by *Man2c1* suppression, as few papers dealing with the possible functions of Man2c1 can be found in the literature,[24] it is impossible to figure out an exact picture. Based on the fact that the major function of α-mannosidase is to trim mannoses in N-glycans[25] and most proteins, including transcription factors, are N-glycosylated,[26,27] we imagine that *Man2c1* suppression might result in N-glycosylation modification of some transcription factors, consequently influencing transcription of genes including *BCSC-1*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting information: Materials and Methods

Fig. S1. Sequence alignments for eight homologues of BCSC-1 proteins.

Fig. S2. *Man2c1* suppression caused upregulation of *BCSC-1* in CNE-2L2 cells.

Fig. S3. *BCSC-1* expression in human nasopharyngeal carcinomas.

Table S1. List of the oligonucleotide sequences used

Table S2. Genes differentially expressed in AS relative to wild-type CNE-2L2 cells

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