**Susd2/SUSD2, a putative tumor suppressor gene, inhibits growth of colorectal cancer cells**

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**Abstract**

We constructed inducible expression system of **Susd2** gene which we previously identified as a novel tumor suppressor gene. Here we report the growth inhibition of colorectal cancer HCT116 cells and fibrosarcoma HT1080 cells by inducible expression of Susd2 protein from HCT/G5Susd2 and HT/G5susd2 cells by infection of Ad/G4VP2, as revealed by colony formation and growth behavior. Thus, we found that Susd2 inhibits growth of cancer cells and this information may benefit to patients with malignant diseases.

**Keywords:** Tumor suppressor gene, Susd2/SUSD2, TS

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**Introduction**

In recent years globalization has influenced Japanese people in every way especially food habit, which in turn increased incidence of colorectal cancer in men and women, more than 50,000 people dying each year and these numbers increases each year\(^{1}\). One of the most difficult problems of cancer medicine is the recurrence and metastasis of drug-resistant tumor cells. Thus novel efficacious medicines are urgently expected.

Two decades of cancer research revealed the existence of “Cancer Stem Cells, CSCs” as a minor population of tumor cells\(^{2}\). Because of the intrinsic stem cell-like properties of CSCs, this subpopulation of tumor cells is believed not only to initiate and sustain tumor growth, recurrence and metastasis but also mediate chemoresistance\(^{2,3}\).

We recently reported a novel mouse gene **Susd2**, which suppresses growth of human cervical cancer HeLa cells\(^{4,5}\). In the present study we show the construction of inducible expression system for the mouse gene **Susd2** and the suppression of growth of human colon cancer HCT116 cells and human fibrosarcoma HT1080 cells by Susd2 as obtained by colony formation assay and growth properties. This property of Susd2 gene could be applied to future gene therapy of colorectal cancer.

**Materials and Methods**

**Cell culture.** Human embryonic kidney 293 (HEK293), human fibrosarcoma HT1080, HT/G5Luc, HT/G5susd2, human colon cancer cells, HCT116, HCT/G5Luc clone A5, HCT/G5susd2 clone B5-4 cells were cultured as described previously\(^{4,5}\).

**Construction of vectors and recombinant adenoviruses.** Construction of vectors and recombinant adenoviruses, Ad/G4VP2, Ad/G4Luc and Ad/G4susd2 were described previously\(^{6}\).

**Establishment of Susd2-inducible cell lines.** Establishment of Susd2-inducible cell lines, HT/G5Luc, HT/G5susd2, HCT/G5Luc and HCT/G5susd2, were described previously\(^{4,5}\).

**Colony formation assay.** HCT116 and HCT/G5susd2 cells were plated at 3×10\(^4\) cells/60mm plastic dishes and cultured for 2 weeks, followed by fixation and staining with crystal violet and number of colonies were counted.

**Growth of cells.** HT/G5Luc, HT/G5susd2, HCT/G5Luc clone A5, HCT/G5susd2 clone B5-4 cells were plated at 2×10\(^3\) cells/well of 96well plates and growth of the cells were followed by WST method on days indi-
Detection of Susd2 protein. Susd2 protein induced in cells was detected by immunofluorescence or Western blotting, as described previously.

Results

The human SUSD2 gene is localized on chromosome 22q11-q12, and the protein of 820 amino acids contains four major domains, Somatomedin B, AMOP, vonWillebrand factor type D and Sushi (SCR repeat) (Fig. 1), which are shared with other adhesion molecules. In addition, it contains signal peptide of 25 amino acids at the N-terminus, transmembrane region and C-terminal peptide of 16 amino acids extending into the cytoplasm. Susd2 protein is expressed on the plasma membrane as a single transmembrane-spanning type I membrane protein. SUSD2 is expressed in a number of normal human tissues most prominently in lung and kidney (Fig. 2), implicating its important role in normal tissues, with reference to mesenchymal stem cells expressing SUSD2 as their specific marker.

Susd2 suppresses growth of colorectal cancer HCT116 cells. As shown in our previous report, transfected Susd2 gene appeared to inhibit the growth of Kras-transformed mouse NIH3T3 fibroblast, Ki3T3 cells. Thus, we proceeded to construct an inducible expression system of Susd2. As shown in Fig. 3, an inducible expression system was constructed (TSTA, Two Step Transcription Amplification): Susd2 cDNA was inserted in a vector under the control of yeast gal4·VP2 promoter, which was then transfected to HCT116 cells to obtain HCT/G5Susd2 cells possessing Susd2 gene in a cryptic state. When HCT/G5Susd2 cells were infected.
with a recombinant adenovirus expressing gal4VP2 transactivator, Susd2 mRNA is transcribed and Susd2 protein is translated at any time and any amount when needed. Thus Susd2 protein is inducibly expressed by infecting increasing moi of AdG4VP2, as shown by immunofluorescence and Western blotting (Fig. 4). It is imperative that background expression level of Susd2 is practically null, since even small amount of Susd2 suppresses growth of the cells. Infection of HCT/G5Susd2 cells with AdG4VP2 showed a dose-dependent inhibition of colony formation (Fig. 5). Furthermore, quantitative measurement of growth of HCT/G5Susd2 cells by WST method showed again a dose-dependent inhibition of growth by AdG4VP2 infection (Fig. 6d). The same result

Fig 3. Construction of inducible expression system of Susd2 by TSTA. Construction of inducible system of Susd2 is described in Sugahara et al. Susd2 cDNA is inserted in pG5 vector under the control of yeast Gal4 promoter as depicted (pG5/Susd2). HCT116 cells harboring the vector was established by transfecting the vector and selected for the clone expressing Susd2 (HCT116/Susd2, B5-4 clone). Susd2 is expressed inducibly by infection of recombinant adenovirus expressing Gal4-VP16 hybrid transactivator (AdVP2), Two-Step –Transcription Amplification (TSTA).

Fig 4. Inducible expression of Susd2 by infection of increasing moi of AdG4VP2. On the left are shown immunofluorescence pictures of B5-4 cells infected with increasing moi of AdG4VP2. On the upper right is shown a picture of HEP293 cells transfected with recombinant adenovirus AdSUSD2. On the lower right is shown Western blot of B5-4 cells infected with AdG4VP2 as above.
was shown with HT/G5Susd2 cells, human fibrosarcoma cells expressing Susd2 (Fig. 6b). Of note, it is an important finding that the same amount of infecting AdG4VP2 virus had no effect on the growth of either HCT/G5Luc or HT/G5Luc cells expressing luciferase (Fig. 6a, c).

Fig 5. Inhibition of colony formation of HCT116/Susd2 cells (B5-4) by infection of AdG4VP2. B5-4 cells subconfluent in 60 mm dish were infected with increasing moi of AdVP2, harvested and plated. Two weeks after plating cells were stained with crystal violet and number of colonies counted. On the left panel were shown colonies in control plate (top), low moi (n=4) plate (middle) and high moi plate (n=3) (bottom). On the right panel is shown histogram of ratios of colony formation efficiency upon increasing moi of AdVP2 infection.

Fig 6. Growth inhibition of tumor cells by Susd2. HT1080/Luc (a), HT1080/Susd2 (b), HCT116/Luc (c) and HCT116/Susd2 (d) cells were infected with increasing moi of AdVP2 on day 1 after plating and growth of the cells was followed by WST method on days indicated after infection. Methodology of WST is detailed in the text.
Discussion

Most serious problem in cancer clinics is frequent reccurrence and metastasis by drug-resistant cancer cells after long period of treatment, which leads to cancer death. New treatment modalities of cancer emerge, molecular targeted therapeutics, immuno-check-point inhibitors etc. However, five-year survival rate of cancer patients remains practically unchanged. Therefore those who overcome drug resistance and decrease metastasis may prevent cancer death. Cancer cells frequently acquire resistance against conventional low molecular weight chemotherapy sooner or later after treatments. There have been a number of attempt to use tumor suppressor genes such as p53 for gene therapy with little success\(^{(15)}\).

In search for new agent for gene therapy we found Susd2 gene in Kras-transformed mouse fibroblast NIH3T3, Ki3T3. Susd2 comprises 820 amino acids expressed on the plasma membrane in many normal tissues of mouse and humans, and the function of Susd2 is still elusive. Expression of SUSD2 is low in various cancer tissues such as ovary\(^{(10)}\), liver\(^{(11)}\), kidney\(^{(12)}\), non-small cell lung\(^{(13)}\) and colorectal cancer cells (unpublished data). An inverse correlation was found between SUSD2 expression level in these cancer tissues and prognosis of the patients. We also found the low expression of SUSD2 in colorectal cancer cell lines and colorectal cancer tissues (unpublished data).

In summary, in the present study we show the construction of Susd2 inducible expression system, whereby we can investigate functional characteristics of Susd2 protein in cancer cells. Thus we found that Susd2 inhibits growth of colorectal cancer HT116 cells and fibrosarcoma HT1080 cells. Therefore, Susd2/SUSD2 gene or protein may suppress the growth of malignant cells in vitro. This evidence may have a great benefit in patients with cancer. Patents of the gene Susd2 were assessed by Japan Patent Adminidration\(^{(16)}\) and the Patent Office of the USA\(^{(17)}\).

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