主論文の要旨

SATB2 suppresses the progression of colorectal cancer cells via inactivation of MEK5/ERK5 signaling

（SATB2 は MEK5/ERK5 シグナルを不活性化することで
大腸がん細胞の浸潤を抑制する）

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<Background>

Special AT-rich sequence-binding protein 2 (SATB2) is a transcription factor that specifically binds to the nuclear matrix attachment region (MAR) of DNA to regulate chromatin remodeling and transcription. Accumulating evidence has revealed that SATB2 has multiple roles in craniofacial patterning, brain development, and osteoblast differentiation. Recent studies have indicated that lower expression of SATB2 is associated with cancer progression and advanced clinical staging. In colorectal cancer, high SATB2 expression is associated with a favorable prognosis and sensitivity to chemotherapy and radiation. Also, the expression of microRNA-31 (miR-31) is correlated with an unfavorable prognosis in colorectal cancer patients, and miR-31 expression was found to promote an aggressive cancer cell phenotype. SATB2 is a direct target of miR-31, and the expression of SATB2 could attenuate the tumorigenicity induced by miR-31. These studies indicate a possible role of SATB2 as a tumor suppressor gene; however, a detailed analysis of SATB2 function in cancer cells has not been fully performed. In this study, we examine the effect of SATB2 expression in colorectal cancer cells. We show here that the exogenous expression of SATB2 represses the aggressive phenotype of colorectal cancer cells, which is partly mediated by the inactivation of extracellular signal regulated kinase 5 (ERK5).

<Materials and Methods>

Colorectal cancer samples and normal colorectal tissues were obtained from patients who underwent surgery at Nagoya University Hospital in 2012. Relative mRNA expression was measured by quantitative RT-PCR analysis. HCT116, HT29 and DLD-1 cells were authenticated and maintained in suitable media. Retrovirus infection was used for ectopic expression of SATB2, deletion constructs, and Constitutive active and dominant-negative forms of MEK5. MTT and colony formation assays were conducted to assess the proliferation and anchorage-independent growth respectively. Wound healing and Matrigel-coated boyden chamber assays were conducted to evaluate the migration and invasion respectively. For *in vivo* experiment, xenograft tumor assay was conducted using male nude mice.

<Results>

SATB2 mRNA levels were reduced in 21 human colorectal cancerous specimens, with 15 samples showing more than 5-fold decreases in SATB2 mRNA compared with the normal controls (Figure 1A). Then, we established HCT116 and DLD-1 cells that constitutively expressed GFP or GFP-SATB2 by retrovirus infection. The level of exogenously expressed GFP-SATB2 was significantly higher than that of endogenous SATB2 (Fig. 1B). Ectopic expression of SATB2 in colorectal cancer cells attenuated the proliferation (Figure 1C), anchorage-independent growth (Figure 2A), *in vivo* xenograft tumor (Figure 2B & 2C) and spheroid formation (Figure 2D). These results show that SATB2 has a suppressive effect on

cancer proliferation both *in vitro* and *in vivo*. In addition, reductions in both migration and invasion due to SATB2 expression were observed using HCT116 cells (Figure 3A &3B).

SATB2 has two independent characteristic domains: the homeobox domain and CUT domain. The CUT domain is a DNA-binding domain that is often found with a homeobox domain. There are two tandem CUT domains followed by one homeobox domain in SATB2. Thus, we created the deletion constructs depicted in Figure 4A and investigated which regions are essential for the suppressive function of SATB2. An immunoblot analysis confirmed the expression of each construct at the expected molecular weight (Figure 4B). FL (full length), ΔCUT, and ΔHOX accumulated in the nucleus, whereas ΔN localized to both the nucleus and cytoplasm, indicating that the N-terminal portion is important for SATB2 accumulation to the nucleus (Fig. 4C). Cell proliferation and colony formation assays revealed that ΔHOX, but not ΔCUT or ΔN, could suppress the proliferation, anchorage-independent growth, migration and invasion of HCT116 cells (Figure 4D, 4E, 4F & 4G).

The phosphorylation of ERK5 was reduced by SATB2 expression in both HCT116 and DLD-1 cells. This reduction in ERK5 phosphorylation was detected by either a phospho-specific antibody or ERK5 mobility shift (Figure 5A). As shown in Figure 5B, the expression of dominant negative DN-MEK5 suppressed ERK5 phosphorylation but did not affect ERK1/2 phosphorylation. The expression of DN-MEK5 clearly suppressed the anchorage-independent growth, migration and invasion (Figure 5C, 5D & 5E).

We next investigated whether the activation of ERK5 could restore the malignant characteristics of SATB2-expressing cells. To activate ERK5, we used a constitutively active form of MEK5 (CA-MEK5). The active form of ERK5 was clearly increased in the SATB2/CA-MEK5 HCT116 cells (Figure 6A). As shown in Figure 6B, 6C, 6D & 6E, the reduced cell growth due to SATB2 expression was partially restored by ERK5 activation. Together, these results indicate that the tumor-suppressive function of SATB2 is partly mediated by the inactivation of ERK5.

<Discussion>

In this report, SATB2 expression reduced the malignant characteristics of colorectal cancer cells. Together with the previous findings, our results clearly indicate that SATB2 has a tumor suppressive function in colorectal cancer. Our analysis using deletion constructs revealed that the N-terminal half as well as the CUT domains were essential for the suppressive function of SATB2. It was rather surprising that deletion of the homeobox domain did not affect SATB2 function. The homeobox domain binds to conserved DNA sequences to promote the transcription of target genes; therefore, deletion of this domain often disrupts the function of homeobox proteins. It appears that homeobox domain-mediated transcriptional regulation is dispensable for the tumor-suppressive functions of SATB2, whereas binding to the AT-rich MAR sequences for chromosomal remodeling plays a critical role in SATB2 function.

Interestingly, the N-terminal half was also required for SATB2 function and localization to the nucleus. Although the N-terminal region of SATB2 does not have any specific motifs, the region is highly conserved between SATB2 and its homolog SATB1. The N-terminal region of SATB1 has been reported to mediate dimerization of the protein; therefore, the dimerization of SATB2 may be required for proper localization to the nucleus and function.

In addition, we found that SATB2 expression reduced the phosphorylation of ERK5 in colorectal cancer cells. The inactivation of ERK5 attenuated the malignant characteristics of HCT116 cells, and the activation of ERK5 recovered the ability of SATB2-expressing cells to migrate, invade, and grow in soft agar. These results clearly indicate that the inactivation of ERK5 is associated with the tumor inhibitory functions of SATB2. A previous study reported that the expression of microRNAs related to MAPK signaling was attenuated by SATB2 expression; thus, the expression of proteins necessary for ERK5 activation may be reduced by SATB2. In contrast to SATB2, SATB1 has been proposed to be a tumor-promoting factor. Although SATB1 and SATB2 are highly similar at the amino acid level, these two proteins have been reported to have antagonistic functions in embryonic stem cell differentiation. An important area of further research will be to determine how the two proteins exert opposing functions in development and tumorigenesis.

<Conclusions>

In summary, we showed that SATB2 has a tumor-suppressive function in colorectal cancer cells via inactivation of MEK5/ERK5 signaling. Further studies to define chromosome architecture and gene profile changes due to SATB2 expression may reveal interesting features of the tumor-suppressive function of SATB2.