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Alternative splicing variant of NRP/B promotes tumorigenesis of gastric cancer

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Gastrointestinal cancer is associated with a high mortality rate. Here, we report that the splice variant of NRP/B contributes to tumorigenic activity in highly malignant gastric cancer through dissociation from the tumor repressor, HDAC5. NRP/B mRNA expression is significantly higher in the human gastric cancer tissues than in the normal tissues. Further, high levels of both the NRP/B splice variant and Lgr5, but not the full-length protein, are found in highly tumorigenic gastric tumor cells, but not in non-tumorigenic cells. The loss of NRP/B markedly inhibits cell migration and invasion, which reduces tumor formation *in vivo*. Importantly, the inhibition of alternative splicing increases the levels of NRP/B-1 mRNA and protein in AGS cells. The ectopic expression of full-length NRP/B exhibits tumor-suppressive activity, whereas NRP/B-2 induces the noninvasive human gastric cancer cells tumorigenesis. The splice variant NRP/B-2 which loses the capacity to interact with tumor repressors promoted oncogenic activity, suggesting that the BTB/POZ domain in the N-terminus has a crucial role in the suppression of gastric cancer. Therefore, the regulation of alternative splicing of the NRP/B gene is a potential novel target for the treatment of gastrointestinal cancer. [BMB Reports 2022; 55(7): 348-353]

INTRODUCTION

Alternative splicing is one form of gene processing that leads to protein diversity (1). Approximately 95% of human multi-exon genes are alternatively spliced (2). Alternative splicing allows the production of numerous proteins from one pre-mRNA, which leads to very high efficiency of protein manufacturing in cells. However, there are some disadvantages to alternative splicing. Among them, is the association of alternative splicing with cancer development (2-6). Generally, eight common traits are needed for cancer development (7): resistance to cell death (8), proliferative signaling (9), acquisition of the ability to invade normal tissue and metastasize (10), the induction of angiogenesis (11), the enabling of replicative immortality (12), the reprogramming of energy metabolism (13), the evasion of growth suppressors (14), and the avoidance of immune destruction (15). Various isoforms of the hyaluronic acid receptor, CD44, are generated by alternative splicing and some isoforms are known to impact the key features of cancer cells, such as metastasis or tumor-initiating potential (16). For example, the abundance of CD44 variant isoforms containing exon V6 increases in colorectal carcinoma as the disease progresses to the later stages in which metastases occur.

NRP/B, also known as ENC1 or PIG10, characterized as a nuclear matrix protein, is implicated in neuronal differentiation and the malignant tumorigenesis of various cancers, such as medulloblastoma, prostate cancer, glioblastomas, astrocytomas, and colon cancer (17-21). NRP/B contains two structural domains: the N-terminus BTB domain and six repeats of a Kelch domain in the C-terminus (22, 23). NRP/B has two isoforms named 57-kDa and 67-kDa by alternative splicing, which were first detected in primary rat neurons and rat and human neuroblastoma cells (17, 20). Although there is much evidence of the abnormal NRP/B expression in many human cancers, the molecular mechanism of each NRP/B isoform on tumorigenesis has not been elucidated. Moreover, the relationship between the expression pattern of NRP/B in gastric tissue and tumorigenesis has not yet been identified.

We have reported the dual function of NRP/B as a tumor suppressor and oncogenic factor, and that the switching between those fates occurs through an alternative splicing process (24). In addition, loss of the BTB/POZ domain in the NRP/B gene by alternative splicing disrupted the interaction of NRP/B with repressor complexes, pRb and HDACs, leading to increased tumorigenesis (24). In this study, we found that the expression of NRP/B was higher in gastric cancer tissue than in the normal tissue, and in particular, the expression of the 57-kDa NRP/B

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was higher than that of the 67-kDa NRP/B. Therefore, we hypothesized that targeting the alternative splicing of NRP/B could be a potential therapeutic approach for gastric cancer. The NRP/B expression was higher in AGS cells than in the SNU638 and SNU668 cells, even though all the cells were derived from gastric cancer. In addition, there was a stronger expression of 57-kDa NRP/B than 67-kDa NRP/B in the AGS cells, compared with similarly low expression of the two isoforms in SNU638 and SNU668 cells. The different expression patterns of NRP/B contribute to the cell tumorigeneses processes, such as migration, invasion, and colony formation; the depletion of 57-kDa NRP/B in AGS cells suppressed the mechanisms of tumorigeneses, whereas the overexpression of 57-kDa NRP/B induced tumorigenesis in SNU638 cells. These implied that the alternative splicing of the NRP/B gene regulates gastric cancer tumorigenesis.

RESULTS

NRP/B expression in human gastric cancer tissues

The expression of NRP/B in human gastric cancer tissues was examined in the biopsies of the enrolled subjects. The NRP/B proteins were expressed throughout the malignant lesions and showed a higher level of expression than in normal tissues (Fig. 1A). NRP/B has two isoforms, "57 kDa" and "67 kDa", which play contrasting roles in several biological processes (25). In a previous study, two NRP/B protein species were immunologically detected in rat and human neuroblastoma cell lines, whereas RNA from each of the two species was not detected in any cell line (17). Therefore, we constructed three primers for two forward primers and one reverse primer to identify the difference in gene expression between the encoded isoforms of the NRP/B gene (Fig. 1B). The alternative splicing of the NRP/B gene induces differences in the mRNA detection due to the specificity of the primer design. Primer 1 doesn't detect NRP/B mRNA, whereas primer 2 binds to the exon II region and detects NRP/B mRNA (Fig. 1B). In accordance, we assessed the difference in the mRNA expression pattern in gastric cancer tissues compared to normal tissues (Fig. 1C-E). The DNA gel yielded two bands: the NRP/B full-length fragment (NRP/B-1: p67-NRP/B) and the isoform fragment (NRP/B-2: p57-NRP/B) where alternative splicing occurred (Fig. 1C). The expression of total NRP/B genes including the full-length NRP/B was strongly increased in gastric cancer tissues. Similarly, the NRP/B-2 gene was detected in gastric cancer tissues, whereas it was hardly detected in normal tissue samples (Fig. 1C-E). These results indicated that NRP/B-2 gene expression was increased in gastric cancer tissues and was an indication of an unfavorable prognosis.

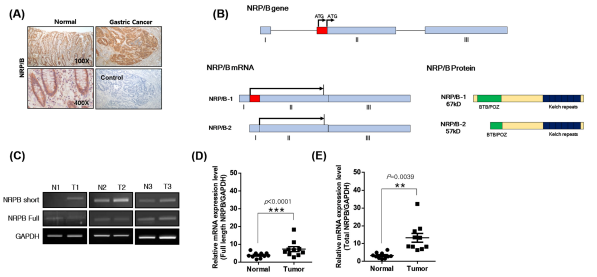

Fig. 1. NRP/B is expressed in gastric cancer tissue. (A) NRP/B expression in human gastric tissue. (B) The genomic structure of the NRP/B gene and the two isoforms of NRP/B were inferred from the expressed sequence data. Exons are shown as blue boxes, and the alternatively spliced exonic region of exon II is colored red. The alternative start codon results in the generation of two isoforms, the shorter (NRP/B-2) of which is missing a portion of the BTB/POZ dimerization domain. The protein-coding region for each isoform is represented by a black arrow. (C) The expression of NRP/B mRNA analyzed by semi-quantitative PCR in gastric cancer tissues. (D) The mRNA expression of the NRP/B-1 in gastric cancer was measured by RT-qPCR. (E) The mRNA expression of the NRP/B-2 in gastric cancer was measured by RT-qPCR. NRP/B-1: full-length p67-NRP/B; NRP/B-2: variant p57-NRP/B. **P < 0.01; *** P < 0.005.

Alternative splicing of NRP/B regulates tumorigenesis in gastric cancer cells

To investigate the correlation between the alternative splicing of NRP/B and the gastric cancer tumorigenesis, we measured NRP/B expression in gastric cancer cells. Both SNU638 and SNU668, diffuse-type cancer cell lines, had lower expression levels of NRP/B-2 than NRP/B-1 (Fig. 2A-C). In contrast to the SNU638 and SNU668, AGS which is an intestinal-type gastric cancer cell line had significantly higher expression levels of

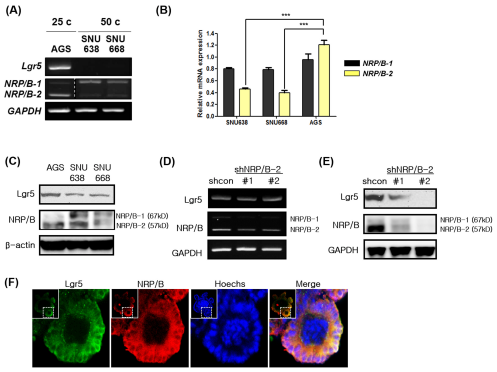

Fig. 2. The expression of the NRP/B isoform in gastric cancer cells. (A) The mRNA expression of NRP/B and Lgr5 analyzed by semi-quantitative PCR in gastric cancer cells. (B) The mRNA expression of NRP/B isoforms in gastric cancer cells analyzed by RT-qPCR. (C) The protein expression of Lgr5 and NRP/B in gastric cancer cells analyzed by western blot. (D) The mRNA expression analyzed by semi-quantitative PCR and (E) the protein expression analyzed by western blot of NRP/B and Lgr5 in shRNA targeting NRP/B-2-(shNRP/B)-transduced clones of AGS compared to control vector (pLV)-transduced AGS cells. (F) NRP/B protein expression analyzed using immunofluorescence staining in the gastric organoid made from mouse gastric stem cells. Lgr5, a gastric cancer stem cell marker, was used as a control. The expression site of NRP/B and LGR5 was shown to overlap in the gastric organoid. *** P < 0.005.

NRP/B-2 (Fig. 2A-C). Based on these results, we hypothesized that alternative splicing of the gene may be an indication of gastric cancer tumorigenesis. To investigate whether p67- or p57-NRP/B is predominantly expressed in gastric cancer, we generated stable NRP/B-2-depleted AGS cells. We confirmed NRP/B-2 knockdown and Lgr5 expression in AGS (Fig. 2C-E). The Lgr5 has been known to be elevated in gastric cancer cells and to regulate tumor progression. Unexpectedly, we found a higher Lgr5 expression in AGS than in the other cells (Fig. 2C), but not in NRP/B-2-depleted cells (Fig. 2D, E). These results showed that NRP/B-2 is correlated with Lgr5 expression. In addition, the expression site of NRP/B and Lgr5 was shown to overlap in the organoid made from mouse gastric stem cells (Fig. 2F). Collectively, we speculated that the Lgr5 expression could be altered by NRP/B, therefore, NRP/B regulates tumorigenesis in gastric cancer cells.

NRP/B-2 induces tumor progression

We examined the effect of NRP/B-2 on tumor progression. The proliferation assay in triplicate for each gastric cancer cell showed that the proliferation rate of SNU638 and SNU668 was lower than that of AGS cells (Fig. 3A). NRP/B-2-depleted cells showed no difference in the proliferation rate compared to the control (Fig. 3A) but showed a different pattern in colony formation (Fig. 3B). The AGS control cells formed holoclone morphology, but the NRP/B-2-depleted AGS cells were similar to that of paraclone morphology (Fig. 3B).

Next, we explored whether the alternative splicing of the NRP/B gene was critical to migration and invasion in human gastric cancer cells. The control-AGS with a higher expression of the NRP/B-2 showed predominant migration and invasion abilities than the SNU638 and SNU668 cells; however, those abilities were abrogated in NRP/B-2-depleted AGS cells (Fig. 3C, D). Collectively, the loss of NRP/B-2 in AGS cells did not affect proliferation but resulted in significantly lower cell migration and invasion rates.

Alternative splicing of the BTB/POZ domain in NRP/B caused a loss of interaction with HDAC5, an epigenetic regulator of tumor repressor genes

To identify the role of alternative splicing on tumorigenesis, the NRP/B-2 construct was inserted in SNU638 cells using a transient transfection system, and the proliferation, migration, and invasion were assessed in NRP/B-2-overexpressing cells (Fig. 4A). The proliferation rate of the NRP/B-2-overexpressing SNU638 cells was higher than that of the control SNU638 cells (Fig. 4B). In addition, the results of the migration and invasion assay showed that NRP/B-2 overexpression increased the migratory properties of SNU638 cells, suggesting that NRP/B-2 promotes advanced cell tumorigenesis (Fig. 4C-E). The overexpression of NRP/B-2 in SNU638 cells increased Lgr5 expression and showed a stronger tumorigenic phenotype (Fig. 4A-E). Taken together, NRP/B-2 is involved in the tumorigenesis of gastric cancer cells and induces tumor progression.

In our previous study, the BTB/POZ domain of NRP/B interacted with HDAC1 and subsequently regulated a breast tumorigenic phenotype through transcriptional repression of the E2F target gene (24). HDAC5 belongs to the HDAC class II family

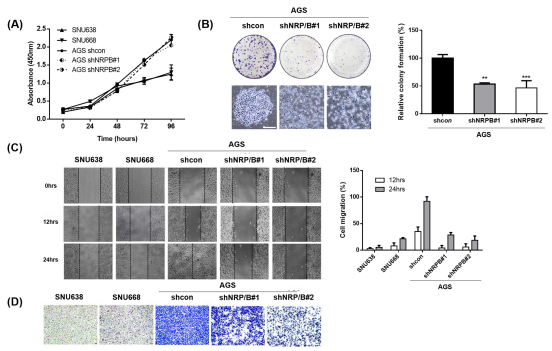

Fig. 3. NRP/B regulates tumorigenesis of the gastric cancer cells. (A) Cell proliferation analyzed by WST assay in the gastric cancer cells and shNRP/B clones of AGS cells. The absorbance was measured using a spectrophotometer every 24-h after initiation. (B) Colony formation implemented to measure the clonogenicity of shNRP/B clones of AGS compared to control AGS cells for 2 weeks. Quantification of colony formation analyzed by the number of colonies. (C) Cell migration toward the gap area was photographed 12 and 24 hours after initiation. Horizontal lines represent the width of the gaps. (D) Representative images from the chamber cell migration assay. The migrated cells were stained with crystal violet. shNRP/B: shRNA targeting NRP/B-2-transduced clones of AGS; shcon: control vector (pLV)-transduced AGS cells. **P < 0.01; *** P < 0.005.

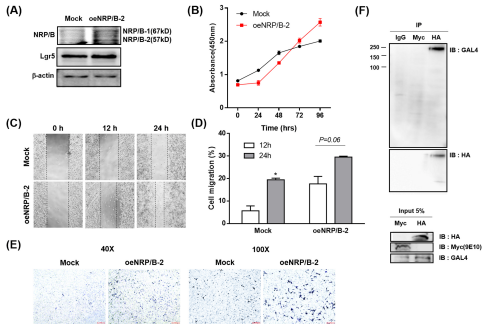

Fig. 4. NRP/B-2 induces tumorigenesis in gastric cancer cells. (A-E) NRP/B-2 is overexpressed in SNU638 cells. (A) The expression of NRP/B and Lgr5 analyzed by using western blotting in NRP/B-2-overexpressing SNU638 cells compared to the control SNU638 cells (Mock). (B) Cell proliferation analyzed by WST assay in NRP/B-2-overexpressing SNU638 cells and compared to control SNT638 (Mock). The absorbance was measured by using a spectrophotometer every 24-h after initiation. (C) Cell migration toward the gap area was photographed 12 and 24 hours after initiation. Horizontal lines represent the width of the gaps. (D) Quantification of cell migration rate. The cell migration percentage was calculated. (E) Representative images from the chamber cell migration assay show the effect of the NRP/B variant form on cell migration after crystal violet staining of migrated cells. (F) Immunoprecipitation of HDAC5 and NRP/B gene.

and is associated with the invasion and metastasis of various cancers, including breast (24), liver (26), and stomach (27). We hypothesized that alternative splicing of the BTB/POZ domain in the NRP/B gene affects the interaction with HDAC5 resulting in differences in the tumorigenic phenotype in the SNU638 and AGS cells. To evaluate our hypothesis, we investigated the interaction between HDAC5, NRP/B-1, and NRP/B-2, and found that HDAC5 binds to NRP/B-1 but not NRP/B-2 (Fig. 4F). This revealed that the alternative splicing of the BTB/POZ could reduce the binding with HDAC5 and that the increased NRP/B-2 in gastric cancer cells could increase the tumorigenic phenotype without altering the expression of HDAC5.

DISCUSSION

The BTB/POZ domain in the NRP/B gene is known to be a key construct for interaction with tumor suppressors. In our study, the NRP/B protein in gastric cancer cells existed as two isoforms, one consisting of the full-length protein (67-kDa) and the other a smaller protein (57-kDa). p57-NRP/B, which results from alternative splicing, has lost the amino terminal portion of the BTB/POZ domain. Many recent studies have identified variations in the transcriptome of tumors; these are caused by the changes induced by alternative splicing, as well as mutations in splicing factors and regulatory signals in most of the tumor types (28). The isoforms of CD44, a transmembrane glycoprotein, were produced by alternative RNA splicing and these variants correlated with aspects of the tumorigenic phenotype, such as cell motility, survival, and proliferation in various types of cancer cells.

In this study, we suggest that the isoform of NRP/B produced by alternative splicing could be a novel marker of gastric cancer diagnosis. Our results showed that NRP/B-2 expression was dramatically increased while NRP/B-1 expression was hardly altered. In addition, the expression ratio of NRP/B-2 to NRP/B-1 was relatively high in gastric cancer tissues, but opposite in nonlesional tissues. This means that the increase in the total NRP/B in gastric cancer tissue is more dependent on NRP/B-2 than on NRP/B-1, indicating that the change in alternative splicing rate is one of the pathogenic factors for gastric tissue tumorigenesis. Interestingly, the cell lines derived from gastric cancer cells showed different NRP/B expression patterns. NRP/B-2 mRNA expression level was higher in AGS, but NRP/B-1 mRNA expression level was lower in AGS that in the SNU638 and SNU668. Consistently, AGS cells showed a predominant ability for proliferation, migration, and invasion than the other cells. NRP/B-2 knockdown in AGS cells significantly reduced colony formation, migration, and invasion, and NRP/B-2 overexpression in SNU638 cells induced an increase in the tumorigenic phenotype. Interestingly, NRP/B-2 knockdown did not affect cell proliferation, but reduced colony formation. This may affect tumor size. In addition, Lgr5 expression regulates colony formation and cell migration through the suppression of ERK phosphorylation in colorectal cancer (29-31). Consistent with this, our results showed that Lgr5 expression was lower in NRP/B-depleted AGS cells than in the control cells, but NRP/B-2-overexpressing SNU638 cells induced an increase in Lgr5 expression.

The function of the BTB/POZ domain in the NRP/B gene is to interact with anticancer transcription factors or proteins (24, 26, 27). Therefore, we speculated that splicing of the BTB/POZ domain negatively regulates complex formation with cancer repressors, resulting in a malignant phenotype. In our previous study, the BTB/POZ domain of NRP/B repressed proliferation and migration in breast cancer cells through complex formation with E2F and HDAC1 (24). HDAC1 generally affects malignant tumor phenotypes via the activation of oncogenes or the inhibition of specific tumor suppressor genes; however, the protein can act as a transcriptional repressor when it interacts with NRP/B (27). HDAC5, part of the HDAC class II family, is known to repress the induction of the tumorigenic phenotype in gastric cancer cells (26). In the current study, NRP/B-1 interacted with HDAC5, but NRP/B-2 could not bind to HDAC5. Since the expression level of NRP/B-1 in AGS cells was relatively lower than that of NRP/B-2, HDAC5 couldn't bind to the BTB/POZ domain of the NRP/B gene. In contrast, in the SNU 638 and SNU668 cells, HDAC5 could readily form a complex with NRP/B-1 which has a BTB/POZ domain. This was proven by the findings that the AGS cells with a low expression of NRP/B-1 displayed an increased tumorigenic phenotype, while other cells with high expression of NRP/B-1 had a relatively low tumorigenic phenotype.

Based on our findings, AGS cells, as an invasive intestinal type of gastric cancer cell, have higher expression of Lgr5 than SNU638 or SNU668 cells. The high expression of Lgr5 in the AGS cells is predictable because the origin of the Lgr5-positive stem cells is an invasive intestinal-type of gastric cancer (32) and the expression of Lgr5 is correlated with an invasive phenotype. Interestingly, regardless of the types of gastric cancer cells, the expression of Lgr5 was decreased in NRP/B-2-depleted cells, but it was increased in the NRP/B-2-overexpressing cells. In addition, NRP/B was colocalized with the Lgr5 expression site in the gastric organoids. These results indicated that the ratio of NRP/B-2 to NRP/B-1 in gastric cancer cells could regulate the expression of Lgr5 and then induce an increase in the tumorigenic phenotype. The correlation between HDAC1 and Lgr5 was confirmed by the reports that HDAC inhibitors could act as anticancer agents in Lgr5-positive cancer cells (30, 33). Although research has shown that Lgr5 was regulated by HDAC1, the correlation between HDAC5 and Lgr5 should not be ignored, because the HDAC inhibitors are used as a universal treatment to the HDAC family; the HDAC family has a similar structure. The decreased formation of NRP/B-HDAC5 complexes resulting from the increase in alternative splicing may affect Lgr5-positive cancer cells' tumorigenesis. Lgr5 was recently identified as a gastric cancer stem cell marker (31). Therefore, further studies are needed to confirm if NRP/B-2 functions as a direct or indirect regulator of Lgr5 and is a marker of gastric cancer stem cells.

MATERIALS AND METHODS

Subjects

Ten patients with gastric cancer participated in this study (Supplementary Table 1). Biopsies were taken from both malignant cancer lesions and non-lesions. Prior consent was obtained from all the participants. This single-center clinical study was performed in accordance with the procedures approved by the institutional review board of Bundang CHA Hospital in Seongnam, Korea (IRB no. 2017-04-018-006).

Statistics

All statistical analyses were performed on raw data using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Comparison groups were analyzed with the unpaired Student *t*-test for parametric distributions while multiple comparisons were analyzed with one-way ANOVA followed by Tukey's post-hoc test. All data are presented as mean ± SEM.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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