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RESEARCH

CD44-SLC1A2 Fusion Transcripts in Primary Colorectal Cancer

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Abstract A CD44-SLC1A2 fusion has recently been discovered in a subset of primary gastric cancers, and an APIP-SLC1A2 fusion has been described in a colon cancer cell line (SNU-C1); however, whether such SLC1A2 fusions occur in primary colorectal cancer (CRC) and whether such fusions are specific for gastrointestinal cancers remain uncertain. In the present study, we examined 90 primary CRCs and 112 primary non-small cell lung cancers (NSCLCs) for CD44-SLC1A2 and APIP-SLC1A2 fusion transcripts using RT-PCR and subsequent sequencing analyses. Although the expression of both types of SLC1A2 fusion transcripts was not detected in any of the NSCLCs, the expression of CD44-SLC1A2, but not the APIP-SLC1A2 fusion transcript, was detected in one (1.1 %) CRC. The CD44-SLC1A2 fusion transcript was expressed in cancerous tissue but not in corresponding non-cancerous tissue, and the fusion occurred between exon 1 of CD44 and exon 2 of SLC1A2; it was expected that a slightly truncated but functional SLC1A2 protein would be produced under the *CD44* promoter. A quantitative RT-PCR analysis revealed that SLC1A2 mRNA expression was upregulated in CRC containing SLC1A2 fusion transcripts, while it was downregulated in most other CRCs. The SLC1A2 fusion-positive carcinoma was located on the right-side of colon, was a mucinous adenocarcinoma, was immunohistochemically negative for MSH2 mismatch repair protein, and contained no *APC* or *KRAS* mutations. Together, these results suggest that the expression of SLC1A2 fusion transcripts is related to a subset of primary CRCs and may contribute to the elucidation of the characteristics of SLC1A2 fusion-positive CRCs in the future.

Keywords CD44-SLC1A2 · Fusion transcript · Colorectal cancer

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Introduction

Fused genes, such as *ALK* and *ROS1* fusions, are important oncogenic drivers in a subset of non-small cell lung cancers (NSCLCs) and are key targets of various agents currently in clinical development.[1–6] Numerous reports on oncogenic fusions in NSCLC have been published to date because of the significant clinical impact of these fusions,[6, 7] but only a small number of papers have discussed recurrent gene fusions in gastrointestinal cancer. Recently, Tao et al. [8] identified a fusion of the *CD44* gene and the glutamate transporter *SLC1A2* gene in a subset (1 to 2 %) of primary gastric cancers. In their paper, both the silencing and the upregulation of CD44-SLC1A2 expression showed that CD44-SLC1A2 enhances cellular proliferation, colony formation, and invasion. Moreover, consistent with the glutamate transporter function of SLC1A2, the accumulation of glutamate, which can act as a growth regulator, was seen in SLC1A2-fusion positive gastric cancer. Notably, CD44-SLC1A2 silencing sensitized cells to a

chemotherapeutic agent. The authors concluded that CD44-SLC1A2 is a pro-oncogenic fusion and a potential drug target. Interestingly, another form of SLC1A2 fusion, APIP-SLC1A2, has been found in a colon cancer cell line (SNU-C1) by another group.[9] However, since no papers other than the two papers mentioned above [8, 9] have been published regarding SLC1A2 fusions, whether SLC1A2 fusions occur in primary colorectal cancer (CRC) and whether they are specific for gastrointestinal cancers has remained unclear. In this study, we examined the presence of CD44-SLC1A2 and APIP-SLC1A2 in a total of 90 CRCs and 112 NSCLCs and further characterized a cancer containing a fusion. This report is the first published study to describe SLC1A2 fusion transcripts in primary CRC.

Materials and Methods

Primary Carcinomas

Samples of surgical specimens from 90 CRC patients and 112 NSCLC patients who had undergone surgery at the Hamamatsu University Hospital and the Mikatahara Seirei General Hospital, respectively, were obtained. The clinicopathological profiles of the CRC cases are shown in Table 1. Regarding the NSCLC cases, the mean age of the 112 patients was 68.1 years (standard deviation, 7.4 years), and they consisted of 85 men and 27 women. The NSCLCs were histologically classified as adenocarcinoma in 61 cases, squamous cell carcinoma in 40 cases, adenosquamous carcinoma in 6 cases, pleomorphic carcinoma in 4 cases, and large cell carcinoma in 1 case. This study was approved by the Institutional Review Boards (IRBs) of Hamamatsu University School of Medicine and Mikatahara Seirei General Hospital.

Table 1 Summary of clinicopathological profiles of colorectal cancer patients

Characteristic	Number
No. of patients	90
Age, years (mean±SD)	63.6±14.3
Gender, n (%)	
Male	52 (57.8 %)
Female	38 (42.2 %)
Histology, n (%)	
Well to moderately differentiated adenocarcinoma	85 (94.4 %)
Poorly differentiated adenocarcinoma	2 (2.2 %)
Mucinous adenocarcinoma	3 (3.3 %)
pT factor, n (%)	
pTis/pT1	6 (6.7 %)
pT2-pT4	84 (93.3 %)
Lymph node metastasis, n (%)	
Negative	44 (48.9 %)
Positive	46 (51.1 %)

SD standard deviation

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted from the tissue samples using an RNeasy kit (Qiagen, Valencia, CA, USA) and was converted to first-strand cDNA using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. PCR was performed in 20-μL reaction mixtures containing HotStarTaq DNA polymerase (Qiagen) under the following conditions: 30 s at 94 °C, 30 s at 61 °C, and 60 s at 72 °C for 45 cycles. The following forward PCR primers were used: 5′-ATG GAC AAG TTT TGG TGG CAC-3′ for the sequence at exon 1 of CD44, and 5′-ATG TCT GGC TGT GAT GCT CG-3′ for the sequence at exon 1 of APIP. The reverse PCR primer used was the same, i.e., 5′-GAA GAA GCC CTC CAC ACA CTG-3′ for the sequence at exon 3 of SLC1A2. The PCR products were fractionated using electrophoresis on an agarose gel and were stained with ethidium bromide. PCR-amplified products were purified with Exo-SAP-IT (GE Healthcare Bio-Science, Piscataway, NJ, USA) and were sequenced directly using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and the ABI 3130 Genetic Analyzer (Applied Biosystems).

Quantitative RT (QRT)-PCR

The expressions of the SLC1A2 mRNA transcripts were measured using real-time QRT-PCR with a LightCycler instrument (Roche, Palo Alto, CA, USA). PCR amplification of the SLC1A2 transcript and the transcript of the control housekeeping gene *GAPDH* was performed with the cDNA and a QuantiTect SYBR Green PCR kit (Qiagen). The following PCR primers were used: 5′-CAG TCA TCT TGG CTC AGA GGA AC-3′ and 5′-GAA GAA GCC CTC CAC ACA CTG-3′ for the SLC1A2 transcript, and 5′-GCT CAG ACA CCA TGG GGA AG-3′ and 5′-TGT AGT TGA GGT CAA TGA AGG GG-3′ for the GAPDH transcript. The T/N ratios were calculated by dividing the normalized transcript amounts in the cancerous tissue by the amounts in the non-cancerous tissue.

Immunohistochemical Staining

Sections of formalin-fixed, paraffin-embedded tissue samples were used for immunohistochemical staining using a Histofine Simple Stain MAX PO kit (Nichirei, Tokyo, Japan), as described previously.[10] The primary antibodies were as follows: anti-CDX2 (Biocare Medical, Concord, CA, USA),

anti-Cytokeratin 7 (CK7) (DAKO, Tokyo, Japan), anti-Cytokeratin 20 (CK20) (DAKO), anti-MLH1 (BD Pharmingen, San Diego, CA, USA), and anti-MSH2 (clone FE11; Calbiochem, San Diego, CA, USA). Hematoxylin-eosin (H&E) staining was also performed.

Mutational Analysis Using PCR Sequencing

Genomic DNAs were extracted from the CRC tissue samples containing SLC1A2 fusion transcripts using a DNeasy kit (Qiagen) and were examined for somatic mutations in the DNA sequences of the entire coding region of the *APC* gene and of mutation cluster regions in the *KRAS* gene. PCR amplification was performed as described previously.[4, 11] Sequencing was performed as described in the "*RT-PCR analysis*" section.

Results

In this study, 90 CRCs and 112 NSCLCs were examined for CD44-SLC1A2 and APIP-SLC1A2 fusion transcripts using RT-PCR and subsequent sequencing analyses. Although the expression of CD44-SLC1A2 or APIP-SLC1A2 was not detected in any of the NSCLCs, CD44-SLC1A2 fusion transcripts, but not APIP-SLC1A2 fusion transcripts, were detected in one (1.1 %) CRC (case No. 55) (Fig. 1a). The SLC1A2 fusion transcripts were expressed in the cancerous tissue but not in the non-cancerous tissue in the CRC case (Fig. 1b). Sequencing of the RT-PCR products revealed that the fusion was between exon 1 of CD44 and exon 2 of SLC1A2 (Fig. 1c). This fusion form was the same as that in a previous report,[8] and protein translation from the fusion transcript initiates from the AUG site within the region corresponding to the *SLC1A2* exon 2.[8] The protein produced from the fusion transcript is 565 amino acids in length and lacks the N-terminal 9 amino acids, when compared with the 574-amino acid-length wild-type SLC1A2; however, it retained all the functional domains such as the transmembrane and symporter domains of SLC1A2.[8] All the above findings suggest that SLC1A2 fusion transcripts are expressed in a subset of CRCs, but not NSCLCs.

Since the CD44-SLC1A2 fusion transcripts are driven by the *CD44* promoter and CD44 is highly expressed in CRC, [12] and, moreover, it was reported that the expression level of SLC1A2 transcripts is increased in CD44-SLC1A2 fusion-positive gastric cancer,[8] we examined the level of mRNA expression of SLC1A2 in CRC. To calculate the ratio of the level of SLC1A2 mRNA expression in the cancerous (T) tissue to the level in the corresponding non-cancerous (N) tissue (T/N ratio), 57 cases with RNA samples of non-cancerous colorectal tissue among 90 cases were analyzed for mRNA expression using a QRT-PCR analysis. An increased SLC1A2 expression (T/N ratio >2) was observed in only 3 (5.3 %) of the 57 primary CRCs, and one of them was a CRC containing CD44-SLC1A2 fusion transcripts (case No. 55) (Fig. 2). On the other hand, reduced SLC1A2 expression levels (T/N ratio

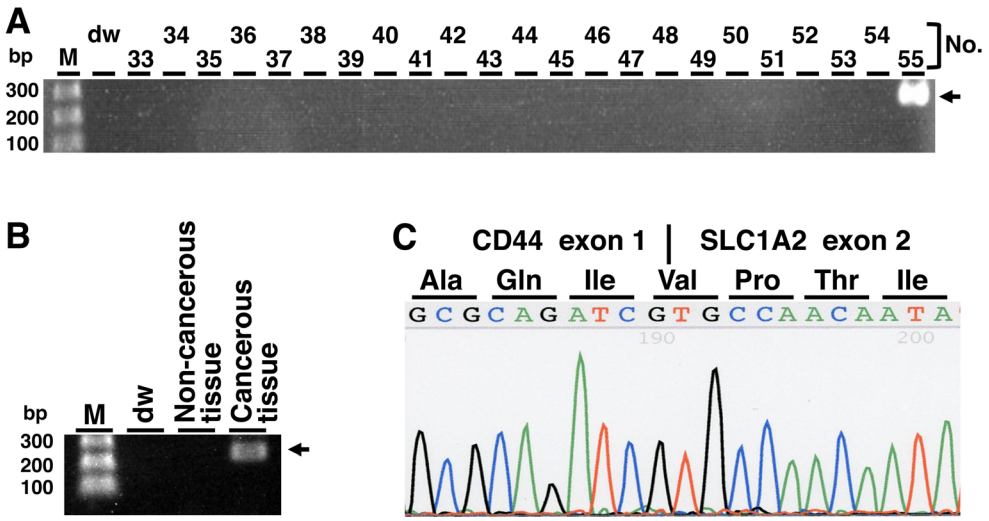

Fig. 1 Detection of SLC1A2 fusion transcripts in primary colorectal cancers (CRCs). **a** cDNA derived from cancerous tissue of primary CRC was searched for SLC1A2 fusion transcripts using reverse transcription (RT)-polymerase chain reaction (PCR) and subsequent agarose gel electrophoresis. A specific band was detected in case No. 55 using PCR with a set of primers for the sequence at CD44 and at SLC1A2; the PCR product band is indicated by the *arrow*. M, size marker. Dw means PCR without cDNA. **b** RT-PCR analysis of cDNA derived from non-cancerous colorectal tissue and colorectal cancerous tissue in case No. 55 for the detection of CD44-SLC1A2 fusion transcripts. PCR products were subsequently subjected to agarose gel electrophoresis. M, size marker. Dw means PCR without cDNA. **c** Sequencing analysis of the SLC1A2 fusion transcripts in case No. 55. A sequencing electropherogram revealed a fusion occurring between exon 1 of CD44 and exon 2 of SLC1A2

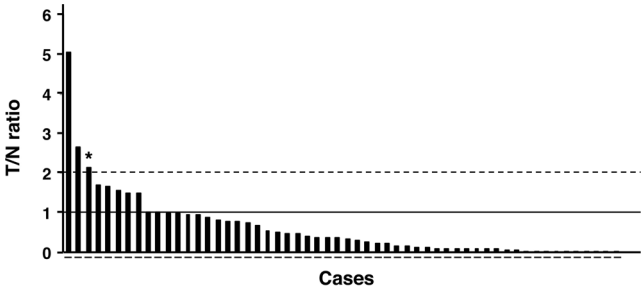

Fig. 2 Comparison between SLC1A2 mRNA expression in cancerous tissues from 57 primary colorectal cancer (CRC) and corresponding non-cancerous colorectal tissues, as determined using a quantitative reverse transcription (QRT)-polymerase chain reaction (PCR) analysis. After normalizing the amounts of SLC1A2 transcripts to those of the GAPDH transcripts, the T/N values were calculated by dividing the amount of normalized transcripts in cancerous tissue by the amount in the corresponding non-cancerous tissue. Cases are lined up in descending order of T/N values. A case with SLC1A2 fusion-positive CRC (case No. 55) is marked by the *asterisk*

<1) were observed in 45 (78.9 %) of the 57 CRCs (Fig. 2). Moreover, a significant difference was detected in the SLC1A2 expression level between the cancerous tissue and the corresponding non-cancerous tissue using a statistical analysis ($P<0.001$ using the Wilcoxon matched pairs test). These results suggested that the upregulation of SLC1A2 mRNA expression is limited to only a small subgroup of primary CRCs, and SLC1A2 fusion-positive CRC is one such subgroup.

Case No. 55, who had a CRC containing CD44-SLC1A2 fusion transcripts, was a 69-year-old man, and the carcinoma in this case occurred in the ascending colon (i.e., the right-side of the colon). Pathohistologically, the carcinoma was a mucinous adenocarcinoma (Fig. 3a and b) that exhibited advanced growth (pT factor: pT4) and metastasized to the lymph nodes (pN factor: pN2). To further characterize the carcinoma, we performed an immunohistochemical analysis and a somatic mutation analysis for the SLC1A2 fusion-positive carcinoma. The immunohistochemical analysis revealed that the colonic adenocarcinoma was positive for CDX2 but was negative for CK7 and CK20 (Fig. 3c–e), meaning that the carcinoma was of colonic origin but had a rare CK7/CK20 immunophenotype, according to a previous paper.[13] Regarding the immunophenotype of two main mismatch repair proteins,[14, 15] the adenocarcinoma was positive for MLH1 but negative for MSH2 (Fig. 3f and g; Fig. 3h shows positive

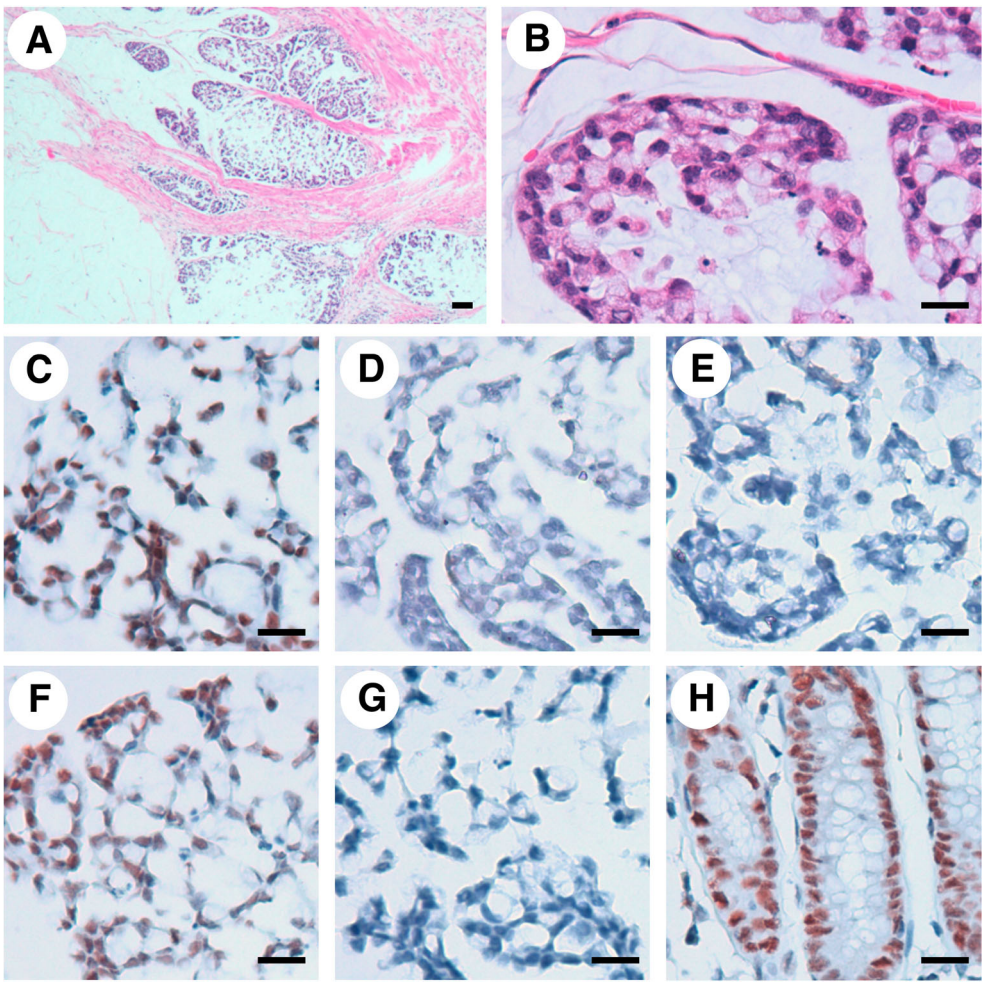

Fig. 3 Immunohistochemical analysis of SLC1A2 fusion-positive colorectal cancer (CRC). **a**, **b** Microscopic photo (H&E) of the SLC1A2 fusion-positive CRC in case No. 55. The histological type of the CRC was mucinous adenocarcinoma. **c**–**h** The adenocarcinoma was immunohistochemically positive for CDX2 (**c**) and MLH1 (**f**) and negative for CK7 (**d**) and CK20 (**e**). MSH2 expression was immunohistochemically negative in the adenocarcinoma (**g**) but was positive in non-cancerous colonic mucosal epithelium (**h**). *Scale bar*, (**a**) 100 μm and (**b**-**h**) 20 μm

MSH2 expression in non-cancerous colonic mucosal epithelium), suggesting that the adenocarcinoma contained defects in the mismatch repair system. The mutation analysis revealed that the absence of somatic *APC* and *KRAS* mutations in the adenocarcinoma.

Discussion

In this study, the expression of SLC1A2 fusion transcripts was found in 1 (1.1 %) of the 90 CRCs, but no expression of SLC1A2 fusion transcripts was detected in any of the 112 NSCLCs that were examined. The fusion occurred between exon 1 of CD44 and exon 2 of SLC1A2; it was expected that a slightly truncated but functional SLC1A2 protein would be produced under the *CD44* promoter. The expression of the CD44-SLC1A2 fusion transcripts was detected in a cancer-specific manner. A QRT-PCR analysis revealed that SLC1A2 mRNA expression was upregulated in the CRC containing the SLC1A2 fusion transcripts, while it was downregulated in most of the other CRCs. The SLC1A2 fusion-containing CRC was a mucinous adenocarcinoma that occurred on the right-side of the colon and exhibited a CDX2 cell lineage, a loss of MSH2 mismatch repair protein expression, and wild-type *APC* and *KRAS* alleles. All the above results suggested that the expression of SLC1A2 fusion transcripts is related to a subset of primary CRCs. As far as we know, this is the first demonstration of SLC1A2 fusion transcripts in primary CRC.

In the present study, the expression of SLC1A2 fusion transcripts was detected at an incidence of 1.1 % in primary CRCs but was not detected in primary NSCLCs. Tao et al. [8] previously reported that its expression was detected in 1 to 2 % of primary gastric cancers. Thus, these results suggest that SLC1A2 fusion is a relatively recurrent event in major gastrointestinal cancers, i.e., gastric cancer and CRC, but not in NSCLCs. However, it is possible that the absence of NSCLCs expressing SLC1A2 fusion transcripts in our series was due to the small number of examined cases. Regarding the fusion partner, CD44 and APIP were screened in this study because other fusion partners with SLC1A2 have not been identified in any tumors. However, since larger numbers of fusion partners have been identified in ALK and ROS1 oncoproteins [16, 17], novel partners of SLC1A2 might be found in the future; in such a situation, the incidence of SLC1A2 fusion might be increased in gastrointestinal cancers.

In this study, the upregulation of SLC1A2 expression was detected in a CD44-SLC1A2-positive CRC. SLC1A2 upregulation was also shown in CD44-SLC1A2-positive gastric cancers in a previous paper [8], and glutamate accumulation, which is consistent with a glutamate transporter function of SLC1A2, was also shown in the fusion-positive gastric cancers in that paper. Increasing evidence suggests that glutamate is an important amino acid necessary for the maintenance of cancer traits. For example, glutamate may provide cancer cells with a route for adenosine triphosphate production, since intracellular glutamate is converted to α-ketoglutarate, an oxidative substrate for the tricarboxylic acid cycle [8, 18]. Additionally, the inhibition of glutamate-initiated signaling pathways decreases growth, invasion, and migration in malignant neoplasms, such as breast cancer, prostate cancer, malignant melanoma, and glioma cells.[19] Thus, SLC1A2 upregulation as a result of a fusion between CD44 and SLC1A2 may confer an aggressive phenotype on CRC cells.

SLC1A2 fusion-positive CRC showed a loss of expression of MSH2 mismatch repair protein in this study. MSH2 is one of two major mismatch repair proteins, and a decreased expression of MSH2 and/or the other protein, MLH1, because of epigenetic changes is a major cause of mismatch repair defects in sporadic CRCs.[14, 15] Approximately 15 % of sporadic CRCs have defects in mismatch repair machinery, and these cancers are frequently found in right-side colon cancer and mucinous adenocarcinoma.[14, 15, 20, 21] In conjunction with the result that location and histology of our case was compatible with the features of mismatch repair-deficient CRCs, all the above results suggest that our SLC1A2 fusion-positive cancer was a mismatch repair-deficient colon cancer. Although whether the mismatch repair-deficient phenotype is common in SLC1A2 fusion-positive CRC is unclear at present, our case may contribute to the elucidation of the characteristics of SLC1A2 fusion-positive CRCs in the future.

In conclusion, the expression of CD44-SLC1A2 fusion transcripts was found in 1.1 % of primary CRCs, and the upregulation of SLC1A2 expression and a mismatch repair-deficient phenotype was also found in the fusion-positive cancer, suggesting that CD44-SLC1A2 fusion is involved in a subset of primary CRCs. Since CD44-SLC1A2 is a potential therapeutic target,[8] our findings might contribute to the selection of a novel subclass that could benefit clinically from SLC1A2 inhibition.

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