Investigation of fusion gene expression in HCT116 cells

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Abstract. Colon cancer is the most common type of gastrointestinal cancer. A number of specific and sensitive biomarkers facilitate the diagnosis and monitoring of patients with colon cancer. Fusion genes are typically identified in cancer and a majority of the newly identified fusion genes are oncogenic in nature. Therefore, fusion genes are potential biomarkers and/or therapy targets in cancer. In the present study, the regulation of specific candidate fusion genes were investigated using Brother of the Regulator of Imprinted Sites (BORIS) in the HCT116 colon cancer cell line, which is a paralog of the fusion gene regulator CCCTC-binding factor (CTCF). The copy number of BORIS increased correspondingly to the progression of colorectal carcinoma from the M0 to the M1a stage. It was identified that EIF3E(e1)-RSPO2(e2), EIF3E(e1)-RSPO2(e3), PTPRK(e1)-RSPO3(e2), PTPRK(e7)-RSPO3(e2), TADA2A-MEF2B and MED13L-CD4 are fusion transcripts present in the transcriptome of the HCT116 colon cancer cell line. CDC42SE2-KIAA0146 is a genomic fusion transcript, which originates from DNA arrangement in HCT116 cells. BORIS suppresses the expression of EIF3E, RSPO2, PTPRK, RSPO3, TADA2A and CD4 to inhibit the expression of fusion transcripts in HCT116 cells. It was hypothesized that the fusion transcripts investigated in the present study may not be oncogenic in HCT116 cells. As BORIS is not colorectal carcinoma-specific, the fusion genes investigated may be a biomarker assemblage for monitoring the progression of colorectal carcinoma.

Introduction

Colon cancer is the most common type of gastrointestinal cancer. The risk of the colon cancer is associated with lifestyle, inherent cause and colorectal adenoma. Diagnosis of colon cancer includes laboratory endoscopy, biopsy, exfoliative cytopathology and carcinoembryonic antigen (CEA) testing. The CEA test measures the amount of this protein that may appear in the blood of certain people who have certain types of cancer, particularly cancer of the large intestine (colon and rectal cancer). However, high CEA levels do not indicate cancer cell metastasis, as low CEA levels were also detected in patients with metastatic colon cancer. Exfoliative cytopathology is not frequently used in clinics, because an ideal exfoliative sample was difficult to obtain. Specific and sensitive biomarkers or a distinct biomarker assemblage may facilitate the diagnosis and monitoring of patients with colon cancer.

Fusion genes are often identified in cancer and numerous newly identified fusion genes have oncogenic properties (1). Therefore, fusion genes are potential biomarkers or therapeutic targets in cancer (2). Fusion genes are a novel type of gene that are a full or partial fusion of two genes and usually result from chromosomal rearrangements (1). Recently, RNA sequencing (RNA-seq) was applied in the study of transcriptomes and novel fusion genes were identified, including SLC45A3-ELK4 and PAX3-FOXO1, which were ascertained to promote cancer progression (3-5). Using transcriptome sequencing, Seshagiri et al (6) identified IF3E-RSPO2 and PTPRK-RSPO3 in clinical colon cancer samples and predicted that these fusion genes are involved in colon cancer progression.

CCCTC-binding factor (CTCF) was previously revealed to suppress expression of the fusion transcript SLC45A3-ELK4 in prostate cancer by binding to the insulators on the genome between SLC45A3 and ELK4 (7). CTCF binds to enhancers or insulators on chromosomes to inhibit the spread of heterochromatin and regulate gene expression (8,9). As there are ~15,000 binding sites for CTCF in the human genome (10), CTCF may regulate the expression of numerous other fusion genes. Qin et al (11) identified that CTCF regulates the expression of fusion transcripts that are not unique to cancer cells. CTCF is widely expressed in normal tissues, so may not drive the onset and progression of cancer (10). Brother of the Regulator of Imprinted Sites (BORIS) is a paralog of CTCF and is expressed in normal testis, ovary and skin cells; however, it is abnormally expressed in breast, prostate and colon cancer cells (12-17). As BORIS has the same zinc-finger domains as those of CTCF (13), BORIS may bind to CTCF-binding sites and regulate fusion transcripts. Considering the potential effect of BORIS on fusion genes and its carcinogenicity, the regulation of fusion genes identified using RNA-seq by Seshagiri et al (6) was evaluated using BORIS in the HCT116 colon cancer cell line. The copy number of BORIS increased as the colorectal carcinoma progressed from the M0 to the M1a stage.

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stage (www.oncomine.org) (18). The HCT116 colon cancer cell line possesses EIF3E(e1)-RSPO2(e2), EIF3E(e1)-RSPO2(e3), PTPRK(e1)-RSPO3(e2), PTPRK(e7)-RSPO3(e2), TADA2A-MEF2B and MED13L-CD4 fusion transcripts within the transcriptome. CDC42SE2-KIAA0146 is a genomic fusion transcript originating from DNA arrangement in HCT116 cells. BORIS suppresses the expression of EIF3E, RSPO2, PTPRK, RSPO3, TADA2A and CD4 to inhibit the expression of fusion transcripts in HCT116 cells. It was hypothesized that the fusion transcripts investigated here may not have oncogenic functions in HCT116 cells. As BORIS is not colorectal carcinoma-specific, the fusion genes investigated may constitute a biomarker assemblage for monitoring the progression of colorectal carcinoma.

Materials and methods

Cell culture. The human HCT116 colon carcinoma cell line and the K562 chronic myelogenous leukemia cell line were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were seeded at a density of 1x10⁶ into 6-well plates prior to drug treatment. When cells reached 70% confluence, 5 μM 5-aza-2’-deoxycytidine (5-Aza-dC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was applied to treat the cells for 48 h, following which an equal volume was added to re-treat the cells for 48 h. Acetic acid (50%) in water was used as the negative control. RNA from the cells was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA), according to the manufacturer’s protocol.

siRNA silencing and transfection. Negative siRNA and BORIS siRNA were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). BORIS siRNA targeting; forward, 5’-AACGACGAUGCCGAACCAAUU-3’ was used for silencing BORIS. The target of negative siRNA is not homologous with any sequence of the human genome. The BORIS overexpression plasmid (p-BORIS) was obtained from OriGene Technologies, Inc. (Rockville, MD, USA). Cells were seeded onto a 6-well plate for transfection. Lipofectamine® RNAiMAX reagent and Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) were applied to, respectively, transfect the siRNA and the plasmid, and to silence and induce the expression of BORIS in HCT116 cells. RNA was extracted 3 days after transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) for subsequent RT using Transcript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Beijing Transgen Biotech Co., Ltd., Beijing, China). The transcript amount was quantified using RT-qPCR and calculated using the 2^-ΔΔCq method (19). The UltraSYBR Mixture was purchased from cwbioitech (Beijing CWBIO Biotech Co., Ltd., Beijing, China) for RT-qPCR reaction. Primers are listed in Table I. RT-qPCR was conducted using the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.).

Genomic DNA extraction and genomic fusion detection. HCT116 and K562 cell genomic DNA was extracted using the phenol-chloroform extraction method (7). Primers used to test fusion genes are listed in Table I. Taq DNA polymerase purchased from Takara Biotechnology Co., Ltd., Dalian, China was utilized for PCR. HCT116 and K562 genomic DNA were applied as templates to detect the putative fusions. The PCR amplicons were separated by 1.5% agarose gel electrophoresis and visualized by BIORAD ChemiDoc imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Clinical data. The copy number amplification frequency of BORIS in two TCGA colorectal carcinoma datasets was summarized by the cBio Cancer Genomics Portal (cBio-Porta, the date of access is September 24th, 2015) (20,21). Associations between the copy number of BORIS and the progression of colorectal carcinoma were analyzed using Oncomine 4.4 (© 2015 Thermo Fisher Scientific Inc.).

Statistical analysis. All experimental data are presented as the mean ± standard deviation. Data in the TCGA Colorectal 2 dataset exported from the Oncomine database were replotted and the significance was calculated using analysis of variance by the software of SPSS Statistics 17.0.0 (SPSS Inc., Chicago, IL, USA). The significance of up- or downregulation was calculated using Student’s t-test type 2 analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

BORIS copy number alteration in colorectal carcinoma. BORIS is expressed in the testis, ovary and skin cells of healthy individuals (13,22). Prior studies have identified the abnormal expression of BORIS in a number of somatic cancer types, including breast cancer and prostate cancer (15-17,23). Fig. 1A indicates a copy number amplification frequency of ~10% for BORIS in two TCGA colorectal carcinoma datasets, as summarized by cBioPortal (20,21). Fig. 1B indicates that the copy number of BORIS is increased according to M stage progression. The data were analyzed using Oncomine. It was considered that the increased expression of BORIS may be associated with colorectal carcinoma progression.

Genomic fusion gene examination. Seshagiri et al (6) analyzed 70 pairs of colon cancer and adjacent non-cancerous tissues using RNA-seq. This study identified multiple fusion transcripts, including recurrent gene fusions, involving the R-spondin family members RSPO2 and RSPO3 that together occur in ~10% of patients with colon cancer. By fusing with exon one of EIF3E or PTPRK, the expression of RSPO2 or RSPO3 was increased, and the Wnt signaling pathway was correspondingly activated. To investigate the fusion genes in colon cancer, the existence of fusion genes in the genome of HCT116 cells was initially evaluated. Genomic DNA was extracted from HCT116 and K562 cells. The primers listed in Table I were used to determine the presence of the fusion genes. Putative lengths of the amplicons are listed in Table I and Fig. 2. As the K562 cell line is BCR-ABL fusion gene-positive and its genome is unstable (24), it was investigated whether K562 cells possess the same genomic fusion genes that were identified in colon cancer, in order to validate the specificity of the investigated fusion genes in colon cancer. ‘No template’
in Fig. 2 indicates the negative control of PCR, which did not contain template to preclude the contamination of the PCR system. GAPDH was used as a positive control for PCR amplification. The arrow in Fig. 2B indicates the amplicon of CDC42SE2-KIAA0146 from the HCT116 genome. Fig. 2B indicates that a larger band of the ETV6-NTRK3 amplicon was

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Table I. Primers used in the present study.

<table>
<thead>
<tr>
<th>Fusion gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Transcript length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIF3E(e1)-RSPO2(e2)</td>
<td>ACTACTCGCATCGCGCACT</td>
<td>GGGAGGACTCAGAGGGAGAC</td>
<td>155</td>
</tr>
<tr>
<td>EIF3E(e1)-RSPO2(e3)</td>
<td>ACTACTCGCATCGCGCACT</td>
<td>TGGAGGACTCAGAGGGAGAC</td>
<td>205</td>
</tr>
<tr>
<td>PTPRK(e1)-RSPO3(e2)</td>
<td>AAAGCTCGCATCGCGCACT</td>
<td>GCTTCATGCAATCTCTCTCA</td>
<td>226</td>
</tr>
<tr>
<td>PTPRK(e7)-RSPO3(e2)</td>
<td>TGGAGTCAATTGCTCACPCT</td>
<td>GCTTCATGCAATCTCTCTCA</td>
<td>250</td>
</tr>
<tr>
<td>ETV6-NTRK3</td>
<td>AAGCCCCATCAACCTCTCTCA</td>
<td>GCGCCGTGTTGAGCAGCTC</td>
<td>206</td>
</tr>
<tr>
<td>CDC42SE2-KIAA0146</td>
<td>AAGGCCAGATTGAGCTGATG</td>
<td>AAAGCTGAAATCCCGCCTG</td>
<td>188</td>
</tr>
<tr>
<td>TADA2A-MEF2B</td>
<td>GCTCTTGTGGCGGATTATGA</td>
<td>GGAGCTACCTGTTGGAGCCT</td>
<td>152</td>
</tr>
<tr>
<td>MED13L-CD4</td>
<td>GGTGTATGGCGTCTCGATGTC</td>
<td>TCCAAAGGGCTCTTTCTTGA</td>
<td>151</td>
</tr>
</tbody>
</table>

The transcript length indicates the amplicon length achieved using the primers listed. All primers are from Seshagiri et al (6).
BORIS was overexpressed by transfecting the pBORIS plasmid into HCT116 cells. The empty vector was set as the negative control. BORIS overexpression efficiency is indicated in Fig. 4B. The results revealed that the fusion transcripts and fusion parent genes were all downregulated (Fig. 4A). These results were in agreement with those of 5-Aza-dC treatment, and suggested that BORIS suppresses the expression of fusion transcripts by downregulating the parent genes.

Silencing of BORIS upregulates fusion transcripts by promoting the parent genes. To confirm the function of BORIS in the regulation of fusion transcripts, BORIS was silenced in HCT116 cells. Silencing efficiency was ~70% following transfecting siRNA of BORIS into HCT116 cells (Fig. 5B). The expression of EIF3E and PTPRK was upregulated (Fig. 5A). Owing to the potential toxicity of transfection, the expression of RSPO2, RSPO3 and the fusion transcripts were undetected. Therefore, the expression of one fusion transcripts TADA2A-MEF2B and MED13L-CD4 was investigated. As the fusions of TADA2A-MEF2B and MED13L-CD4 were not detected in the genome of HCT116 (Fig. 2B), TADA2A-MEF2B and MED13L-CD4 may be regulated by BORIS in the transcriptome of HCT116 cells. The RNA of siBORIS-silenced HCT116 cells was extracted and reversed-transcribed into cDNA. RT-qPCR to determine the expression of certain transcripts. The results revealed that the fusion parent genes TADA2A and CD4 were upregulated and the fusion transcripts of TADA2A-MEF2B and MED13L-CD4 were upregulated (Fig. 6). These results suggested that the downregulation of BORIS promotes the expression of the investigated fusion transcripts via upregulating the expression of the parent genes.

Discussion

Chimeric fused nucleic acids were classified as fusion DNA in the genome and as fusion RNA transcripts in the transcriptome.
As a rule, fusion products in the genome are formed through DNA rearrangement (26-28). Fusion RNA transcripts in the transcriptome comprised of cis-splicing of adjacent genes and trans-splicing (fusion between genes on different strands of the same chromosome or different chromosomes) (4,7,11,29). Our study revealed that the existence of fusion RNA transcripts was not always accompanied by DNA rearrangements.

As genomic fusion genes are not typically regulated, the type of fusion investigated in the present study was first confirmed to be DNA rearrangement or RNA fusion. CDC42SE2-KIAAO146 was detected in the HCT116 genome (Fig. 2B). A larger band, in comparison with the putative ETV6-NTRK3 amplicon, was detected in HCT116 and K562 cell lines (Fig. 2B). The fusion between ETV6 and NTRK3 is out of the prediction range. In addition, ETV6-NTRK3 is not specific for colon cancer, therefore the regulation of ETV6-NTRK3 was not examined in HCT116 by BORIS. The other fusion candidates investigated were not detected in the genome of HCT116. The appearance of the fusions at the RNA level, whilst a lack of existence at the genomic DNA level, suggests that they are fusion transcripts on transcriptome.

Figure 3. Overall, 5-Aza-dC suppressed the expression of fusion transcripts and the parent genes. (A) 5-Aza-dC suppressed the expression of fusion transcripts and their parent genes. The expression of targets investigated in control samples was set as 1. The expression levels of targets investigated in 5-Aza-dC-treated samples are presented as the normalized transcript amount compared with the control. (B) 5-Aza-dC induced the expression of BORIS. 5-Aza-dC, 5-aza-2'-deoxycytidine; BORIS, Brother of the Regulator of Imprinted Sites.

Figure 4. BORIS overexpression suppressed the expression of fusion transcripts and the parent genes. (A) BORIS overexpression suppressed the expression of fusion transcripts and their parent genes. The expression of targets investigated in control samples was set as 1. The expression of targets investigated in BORIS-overexpression samples are presented as the normalized transcript amount compared with the control. (B) Efficiency of BORIS overexpression. BORIS, Brother of the Regulator of Imprinted Sites.
Two parent transcripts joined to be one fusion transcript. Therefore, the expression of fusion transcripts may be affected by the expression of the parent genes. The regulation of the parent genes was examined in the present study and BORIS was identified to suppress the parent gene expression to inhibit the expression of the fusion transcripts (Figs. 3-6).

The statistical data analyzed using cBioPortal indicated that the copy number of BORIS was amplified significantly in colorectal cancer. However, a rare mutation of BORIS was detected (Fig. 1A) that suggests that high expression levels of BORIS may be associated with colon cancer.

BORIS is the homolog of CTCF. CTCF regulates the expression of fusion RNA transcripts, including SLC45A3-ELK4 and ADCK4-NUMBL chimeric RNA transcripts (7,11). The cis-splicing of adjacent genes was identified to be regulated by the binding events of CTCF to genome. BORIS and CTCF share the same zinc-finger DNA binding domains (13), therefore BORIS may regulate fusion transcripts by binding to similar genomic regions to CTCF. Furthermore, BORIS has a role in chromatin organization and gene expression; it demethylates chromatin to regulate the gene expression of cancer testis antigens, and recruits H3K4 methyltransferase to promote the expression of MYC and BRCA1 (30,31). Thus, BORIS may also regulate fusion transcripts by affecting the expression of the parent genes. In the present study, BORIS was identified to inhibit the expression of EIF3E, RSPO2, PTPRK, RSPO3, TADA2A and CD4 (Figs. 3-6). The underlying molecular mechanism requires further study.

The colon cancer genomic fusion gene CDC42SE2-KIAAO146 was detected in the present study (Fig. 2B). Whether it may be used as target for diagnosis, prognosis and therapy requires further examination in other colon cancer cell lines and clinic samples. The expression of EIF3E(e1)-RSPO2(e2), EIF3E(e1)-RSPO2(e3), PTPRK(e1)-RSPO3(e2), PTPRK(e7)-RSPO3(e2), TADA2A-MEF2B and MED13L-CD4 was detected in the HCT116 transcriptome. The expression of the fusion transcripts was affected by the parent genes, which were inhibited by BORIS (Figs. 3-6). High expression of BORIS frequently associated with cancer and BORIS promotes cancer cell proliferation. On the other hand, chimeric fusion nuclear acids were traditionally considered abnormal products of cancer. In addition, it was predicted that BORIS promotes the expression of abnormal chimeric fusions. However, BORIS did not induce or suppress the expression of the tested chimeric fusions in our study. Therefore, the association between cancer and chimeric fusion RNA transcripts needs further determination. Clinical investigation of the copy number alteration of BORIS in colorectal carcinoma patients...
suggested that the BORIS copy number is increased according to M stage progression (Fig. 1B). As BORIS is not colorectal carcinoma-specific, the fusion genes investigated may constitute a biomarker assemblage for monitoring colorectal carcinoma progression.

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