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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-KIAAO146* 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 enhances 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

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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-KIAAO146* 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 linewere cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were seeded at a density of $1x10^5$ 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 TransScript®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 $2^{-\Delta\Delta Cq}$ method (19). The UltraSYBR Mixture was purchased from cwbiotech (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' t-tests type 2 analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

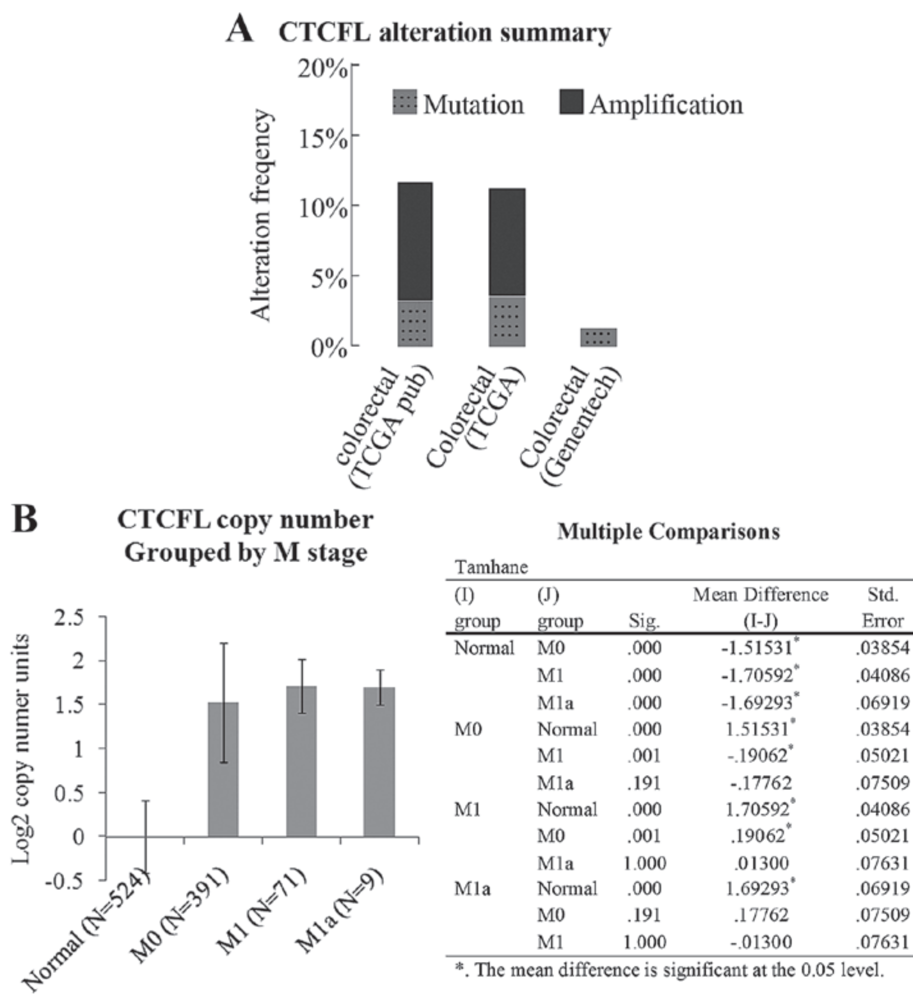
BORIS copy number alteration in colorectal carcinoma. BORIS is expressed in the testis, ovary and skin cells of healthy individuals (13,22). Prior studies have identified the abnormal expression of BORIS in a number of somatic cancer types, including breast cancer and prostate cancer (15-17,23). Fig. 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'

Table I. Primers used in the present study.

Fusion gene	Forward primer (5'-3')	Reverse primer (5'-3')	Transcript length, bp
EIF3E(e1)-RSPO2(e2)	ACTACTCGCATCGCGCACT	GGGAGGACTCAGAGGGAGAC	155
EIF3E(e1)-RSPO2(e3)	ACTACTCGCATCGCGCACT	TGCAGGCACTCTCCATACTG	205
PTPRK(e1)-RSPO3(e2)	AAACTCGGCATGGATACGAC	GCTTCATGCCAATTCTTTCC	226
PTPRK(e7)-RSPO3(e2)	TGCAGTCAATGCTCCAACTT	GCCAATTCTTTCCAGAGCAA	250
ETV6-NTRK3	AAGCCCATCAACCTCTCTCA	GGGCTGAGGTTGTAGCACTC	206
CDC42SE2-KIAA0146	AGGGCCAGATTTGAGTGTGT	AAACTGAAAATCCCCGCTGT	188
TADA2A-MEF2B	GCTCTTTGGCGCGGATTA	GGAGCTACCTGTGGCCCT	152
MED13L-CD4	GTGTATGGCGTCGTGATGTC	TCCCAAAGGCTTCTTCTTGA	151

The transcript length indicates the amplicon length achieved using the primers listed. All primers are from Seshagiri *et al* (6).



Multiple Comparisons

Tamhane

(I) group	(J) group	Sig.	Mean Difference (I-J)	Std. Error
Normal	M0	.000	-1.51531*	.03854
	M1	.000	-1.70592*	.04086
	M1a	.000	-1.69293*	.06919
M0	Normal	.000	1.51531*	.03854
	M1	.001	-.19062*	.05021
	M1a	.191	-.17762	.07509
M1	Normal	.000	1.70592*	.04086
	M0	.001	.19062*	.05021
	M1a	1.000	.01300	.07631
M1a	Normal	.000	1.69293*	.06919
	M0	.191	.17762	.07509
	M1	1.000	-.01300	.07631

*. The mean difference is significant at the 0.05 level.

Figure 1. Copy number abnormality of *BORIS* in colorectal carcinoma. (A) Data were summarized and counted using cBioPortal. 'Mutation' indicates the mutation ratio of *BORIS* in the genome of clinic samples to the corresponding database on the *x*-axis. 'Amplification' indicates the ratio of the patients who gained numerous copies of *BORIS* in the genome to the corresponding database on the *x*-axis. (B) The TCGA Colorectal 2 dataset, which includes the data from 524 healthy volunteers, 391 M0 stage, 71 M1 stage and 9 M1a colorectal cancer patients, was summarized by Oncomine. The CTCFL copy number was grouped by M stage. CTCFL, CTCF-like. The table presents the multiple comparisons between M stage groups using analysis of variance. Sig., significance; Std., standard.

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-KIAAO146* from the HCT116 genome. Fig. 2B indicates that a larger band of the *ETV6-NTRK3* amplicon was

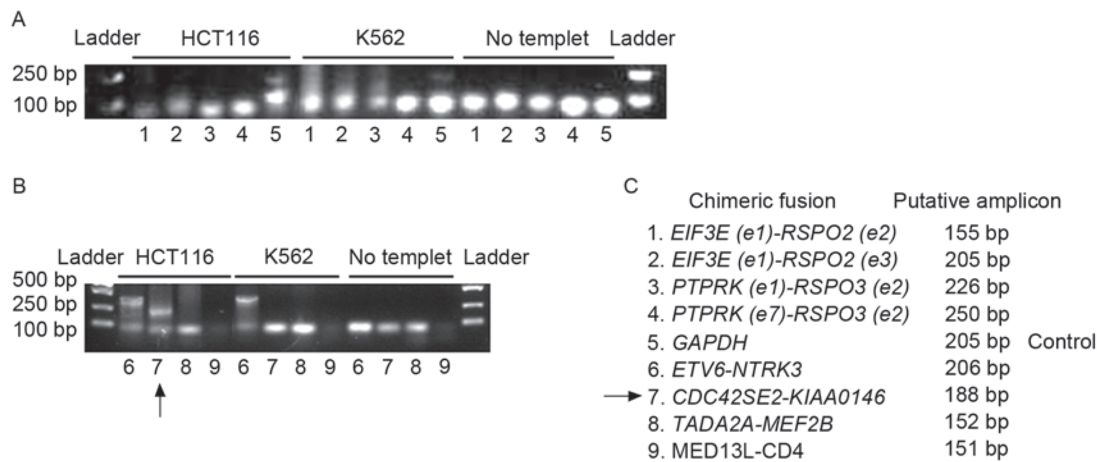


Figure 2. Genomic fusion gene examination. Regular PCR was applied to determine the existence of fusion genes in the genome of HCT116 cells and K562 cells. (A) Detection for the existence of fusions 1 to 5. (B) Detection for the existence of fusions 6 to 9. PCR results were shown by agarose gel electrophoresis. 'No template' indicated the negative control of PCR, which was not supplied with template to preclude the contamination of the PCR system. GAPDH was used as a positive control for PCR amplification. The arrow indicates the amplicon of *CDC42SE2-KIAAO146* from the HCT116 genome. The *ETV6-NTRK3* fusion gene was detected in the HCT116 and K562 genome. (C) The code designation of fusion genes and the putative length of PCR amplicons were listed. PCR, polymerase chain reaction; GAPH, glyceraldehyde 3-phosphate dehydrogenase.

detected in the HCT116 and the K562 genome. The lowest bands on the gels were primer dimers (Fig. 2A and B). The fusion between *ETV6* and *NTRK3* is beyond the range of prediction. Furthermore, the *ETV6-NTRK3* amplicon is not specific for colon cancer (Fig. 2B). Therefore, *ETV6-NTRK3* may not be worthy of further study. Although Seshagiri *et al* (6) demonstrated that fusion genes involving *RSPO2* and *RSPO3* existed in the genome of a limited number of patients with colon cancer, genomic fusion of *EIF3E(e1)-RSPO2(e2)*, *EIF3E(e1)-RSPO2(e3)*, *PTPRK(e1)-RSPO3(e2)*, *PTPRK(e7)-RSPO3(e2)*, *TADA2A-MEF2B* and *MED13L-CD4* was not identified in the genome of HCT116 or K562 (Fig. 2A and B). However, the expression of these fusion genes was detected in the transcriptome of HCT116 (Figs. 3-6).

5-Aza-dC induces the expression of BORIS to downregulate the expression of fusion transcripts. BORIS is the homolog of CTCF and is expressed abnormally in colorectal carcinoma (Fig. 1A). As CTCF regulates the expression of *SLC45A3-ELK4* and other fusion transcriptsin prostate cancer (11), BORIS may also regulate fusion transcripts in colon cancer. In the present study, 5-Aza-dC was used to induce the expression of *BORIS* (14). *BORIS* was upregulated by demethylation, which is induced by 5-Aza-dC treatment (Fig. 3B). The results demonstrated that *EIF3E(e1)-RSPO2(e2)*, *EIF3E(e1)-RSPO2(e3)*, *PTPRK(e1)-RSPO3(e2)* and *PTPRK(e7)-RSPO3(e2)* were suppressed by 5-Aza-dC treatment (Fig. 3A). The expression of *BORIS* was upregulated, and that of *EIF3E*, *RSPO2*, *PTPRK* and *RSPO3* was downregulated. This suggests that 5-Aza-dC promotes the expression of *BORIS* to suppress the expression of the investigated fusion transcriptsand their parent genes (Fig. 3).

Overexpression of BORIS inhibits the expression of fusion transcripts. Although 5-Aza-dC treatment induced the expression of *BORIS*, 5-Aza-dC induced apoptosis (25). 5-Aza-dC may regulate other genes, in addition to *BORIS*, to suppress the expression of fusion transcripts. To exclude this possibility, *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 another two fusion transcripts *TADA2A-MEF2B*and *MED13L-CD4* identified by Seshagiri *et al* (6) in colon cancer was investigated. As the fusions of *TADA2A-MEF2B* and *MED13L-CD4* was 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 was applied 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.

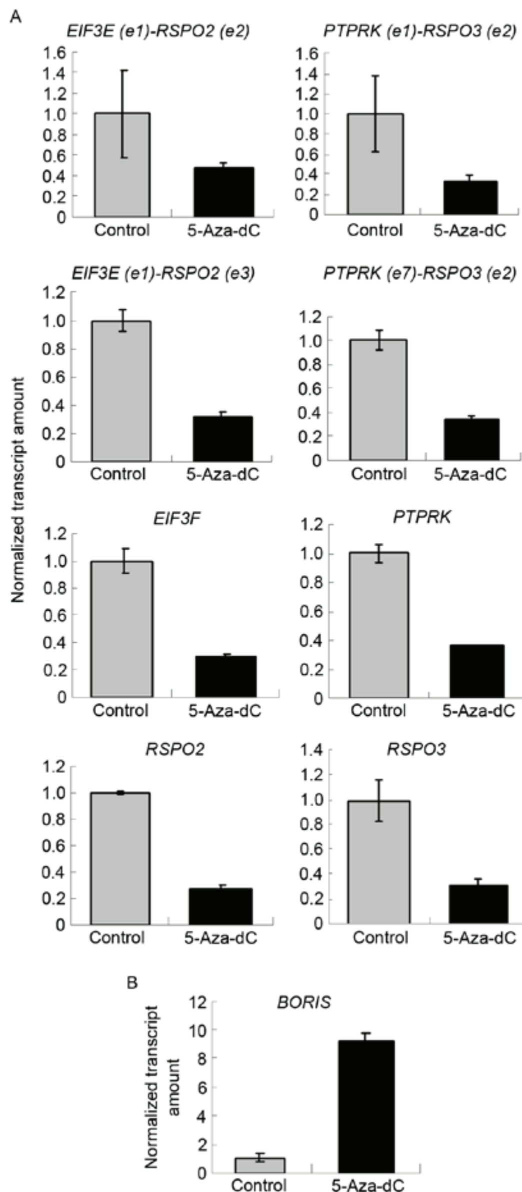


Figure 3. Overall, 5-Aza-dC suppressed the expression of fusion transcripts and the parent genes. (A) 5-Aza-dC suppressed the expression of fusion transcripts and their parent genes. The expression of targets investigated in control samples was set as 1. The expression levels of targets investigated 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.

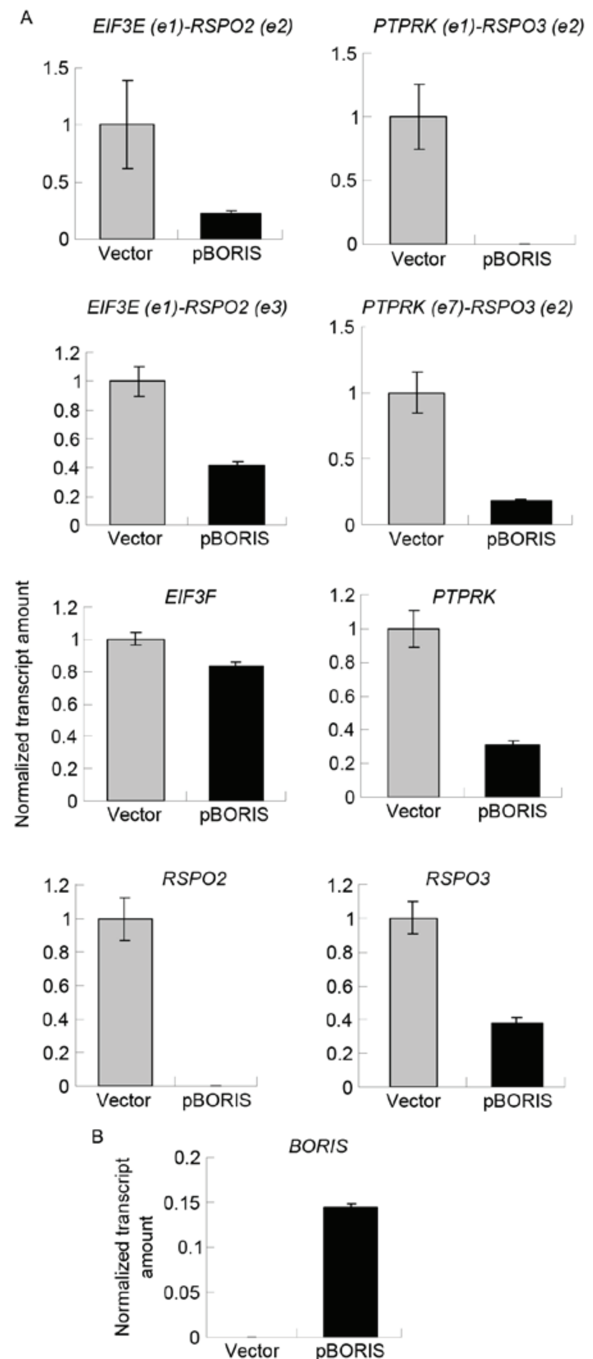


Figure 4. *BORIS* overexpression suppressed the expression of fusion transcripts and the parent genes. (A) *BORIS* overexpression suppressed the expression of fusion transcripts and their parent genes. The expression of targets investigated in control samples was set as 1. The expression of targets investigated in *BORIS*-overexpression samples are presented as the normalized transcript amount when compared with the control. (B) Efficiency of *BORIS* overexpression. BORIS, Brother of the Regulator of Imprinted Sites.

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.

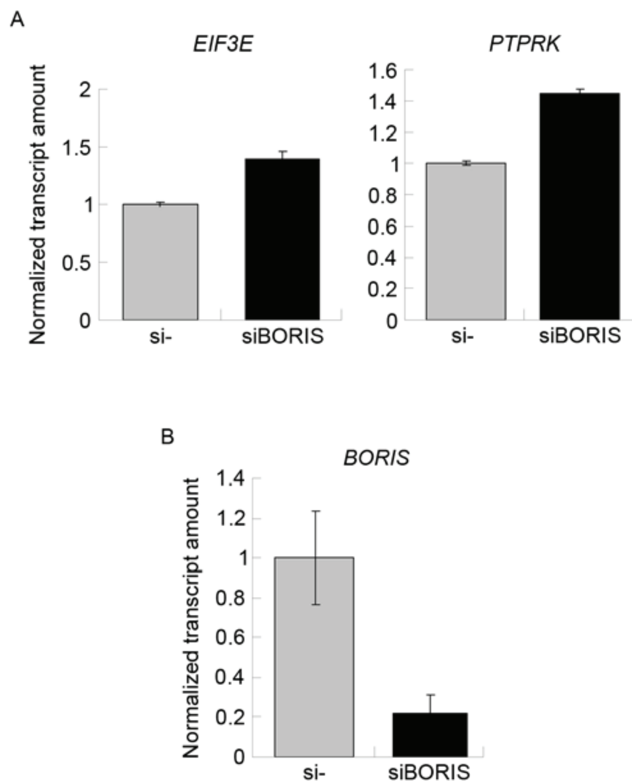


Figure 5. (A) *BORIS* silencing induced the expression of fusion parent genes. The expression of targets investigated in the control samples was set as 1. The expression of targets investigated in *BORIS*-silenced samples are presented as normalized transcript amounts compared with the negative control. (B) *BORIS* silencing efficiency. BORIS, Brother of the Regulator of Imprinted Sites; si, short interfering.

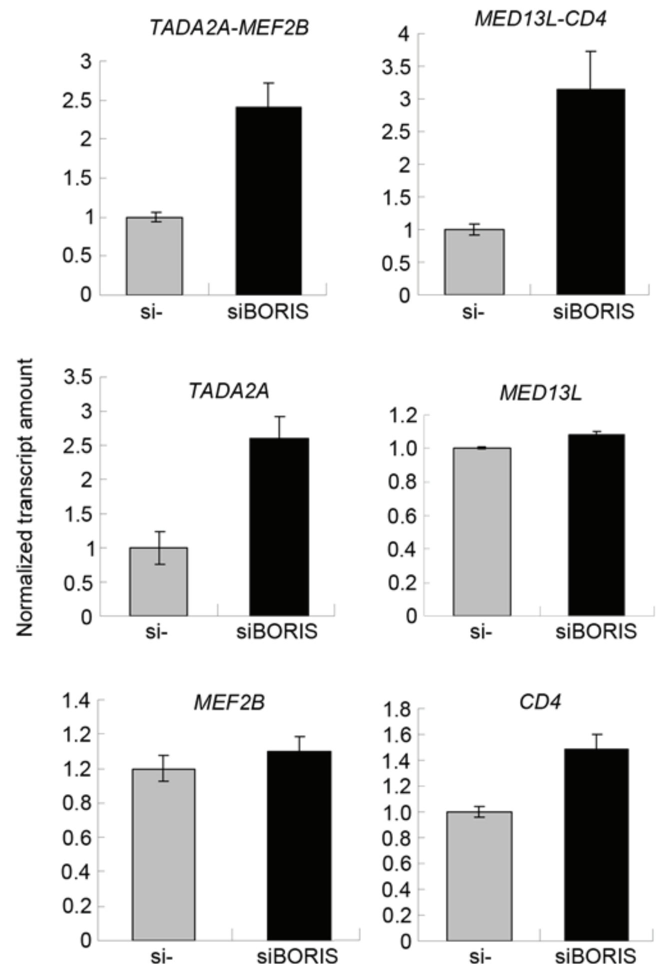


Figure 6. *BORIS* silencing induced the expression of fusion transcripts and their parent genes. The expression of tested targets in control samples was set as 1. The expression of targets investigated in *BORIS*-silenced samples are presented as a normalized transcript amount when compared with the negative control. BORIS, Brother of the Regulator of Imprinted Sites; si, short interfering.

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 indicatedthat 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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