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Antisense RNA transcripts in the blood may be novel diagnostic markers for colorectal cancer

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Abstract. Numerous genetic studies have been conducted regarding the occurrence of colorectal cancer (CRC) and the prognosis using microarrays. However, adequate investigations into the diagnostic application of microarrays have yet to be performed. The simplicity and accuracy of diagnosis and prognosis tracking are important requirements for its processes, and the use of blood cells for diagnosis is considered to be suitable to meet these requirements. The patients involved in the study were 28 preoperative patients with CRC and 6 healthy individuals who served as controls. RNA was extracted from the blood cells of the patients and analyzed using a sense/antisense RNA custom microarray. In the patients with CRC, the expression levels of 20 sense RNA and 20 antisense RNA species were identified as being significantly altered compared with that of the healthy volunteers ($P < 0.05$; fold-change, > 2.0). Cluster analysis of these RNA species revealed that the top 10 antisense RNAs significantly clustered patients with cancer and healthy individuals separately. Patients with stage I or II CRC exhibited significant changes in the expression levels of 33 sense and 39 antisense RNA species, as compared with healthy volunteers ($P < 0.01$; fold-change > 2.0). Cluster analysis demonstrated that patients with stage I or II CRC and healthy volunteers formed separate clusters only among the top 20 antisense RNA species. A tracking study of expression levels of haloacid dehalogenase-like hydrolase domain-containing 1 (HDHD1) antisense RNA was performed and a significant difference was identified between the CRC and healthy

groups revealing that the levels at one week and three months following surgical removal of the cancerous tissue, decreased to almost same levels of the healthy individuals. The results of the current study indicate that HDHD1 antisense RNA may serve as a potential biomarker for the prognosis of CRC.

Introduction

According to the American Cancer Society, colorectal cancer (CRC) is currently the third most malignant cancer in the United States among males and females (1). In the EU, CRC is the second most common cause of cancer-associated mortality in males, following lung cancer, and the third most frequent cause of cancer-associated mortality in females, following breast and lung cancer (2). In Japan, the number of patients with CRC has doubled in the past 20 years to become the second-leading cause of cancer-associated mortality (3).

CRC is a heterogeneous disease that occurs via a complex series of molecular events; a number of genes have previously been demonstrated to have a role in the development of the healthy mucosa of the large intestine into a benign tumor, which then transforms into an invasive cancer (4).

Previous comprehensive studies of $> 13,000$ genes have identified 69 genes that are associated with the pathogenesis of CRC (4), and detailed analysis has revealed that an average of 9 mutant genes are involved in each case of CRC (5). Early CRC detection and the monitoring of patient prognosis are crucial to improve the survival rates of patients with CRC. However, the specimens and array analysis methods reported in previous studies have been problematic for the early diagnosis and monitoring of prognosis, and are not yet suitable for clinical use (4-6).

The development of microarray technology has facilitated the high-throughput analysis of numerous gene expression patterns (5,6). CRC-specific gene expression profiles have been identified in mRNA (5,7-10). Furthermore, the analysis of non-coding RNA, including microRNA (miRNA) and antisense RNA, which cannot serve as templates for direct protein synthesis, has revealed associations between non-coding RNAs and the occurrence of certain types of cancer (11-13). Our previous studies demonstrated the potential involvement

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of antisense RNA expression profiles in the development of CRC and hepatic cancer by examining the expression patterns of specific RNAs in cancerous and healthy tissues (14,15).

Blood samples are ideal for detection of certain types of cancer (12,13) and the monitoring of prognosis; therefore, the present study evaluated RNA expression levels in the blood cells of patients, in order to determine their effectiveness in distinguishing cancerous states and healthy states. The current study identified that certain antisense RNA species in blood cells cluster separately in patients with CRC and healthy volunteers, and revealed that one of these RNA species may serve as a biomarker for prognosis.

Materials and methods

Patients and blood samples. Blood samples were collected from 6 healthy volunteers and from 28 patients with CRC who underwent surgical resection between April 2006 and March 2009 at Tsukuba University Hospital (Tsukuba, Japan). Blood sampling was periodically conducted up until 12 weeks after surgical resection. None of the patients had received radiotherapy or chemotherapy prior to surgery. The primary clinical characteristics for each patient with CRC involved in the current study are presented in Table I. Informed consent was obtained from all patients for the collection of blood samples and the ethics committee of Tsukuba University Hospital approved the study protocol.

Total RNA extraction from blood cells. Blood samples were collected from the patients using PAXgene Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) and subjected to RNA isolation and extraction using a PAXgene Blood RNA Isolation kit (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's instructions. The quantity of total RNA obtained was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the 280/260 nm absorbance ratio, and RNA integrity was evaluated using an RNA 6000 Nano LabChip kit (Agilent Technologies, Inc., Santa Clara, CA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Microarray analysis. Cyanine 3 (Cy3)-labeled cDNA was synthesized from 10 μ g total RNA extracted from the blood samples using a LabelStar Array kit (Qiagen, Inc.) with Cy3-dUTP (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and random nonamer primers. The labeled cDNA was hybridized with probe sequences on an Agilent 44 Kx4 human sense and antisense custom microarray slide (Agilent Technologies, Inc.) (16) in a hybridization solution prepared with an *In Situ* Hybridization Plus kit (Agilent Technologies, Inc.), according to the manufacturer's instructions. The Cy3 fluorescence signals were imaged using an Agilent C DNA microarray scanner (Agilent Technologies, Inc.) and processed using the Feature Extraction version 8.1 software (Agilent Technologies, Inc.).

Statistical analysis. The microarray data were processed using the GeneSpring GX version 12 software (Agilent Technologies, Inc.) to perform the log transformations and the normalization

Table I. Clinical characteristics of patients with colorectal cancer evaluated using microarray analysis (n=28).

Variable	Value
Gender, n	
Male	21
Female	7
Median age (range), years	60 (37-86)
Tumor location, n	
Colon	14
Rectum	14
AJCC stage, n	
I	9
II	9
III	6
IV	4
Other samples, n	
1 week post-surgery samples	4
3 months post-surgery samples	8
Volunteers (non-cancer patients)	6

AJCC, American Joint Committee on Cancer.

of all values to the 75th percentile of the respective microarray, followed by normalization to the respective median expression level for all samples. Additionally, the normalized gene expression data were filtered on flags following the protocol of the manufacturer GeneSpring GX (<http://www.chem.agilent.com/cag/bsp/products/gsgx/manuals/GeneSpring-manual.pdf>). Only those genes classified as flag-present or flag-marginal in >70% of all the array samples, were allowed to pass the filter. The expression profiles of the RNA samples were analysed, using GeneSpring GX version 12 software, with unpaired t-tests, with Benjamini-Hochberg false discovery rate correction (17) for unequal variances, as described in the results section. $P < 0.05$ was considered to indicate a statistically significant difference.

Two-dimensional hierarchical clustering based on Euclidean distance measures was performed using Ward's method (18). The data were visualized using heat maps and dendrograms, as described previously (14). Sample trees were drawn horizontally and gene trees were drawn vertically. Principal component analysis (PCA) was used to identify and characterize trends in multigene expression profiles.

Results

RNA quality. The quality of the RNAs obtained from the blood samples of the healthy volunteers and patients with CRC were initially examined using a NanoDrop Spectrophotometer at an absorbance ratio of 280/260 nm, revealing that the ratios were between 1.8 and 2.0. These results indicated that the total RNAs prepared were usable for labeling with Cy3-dUTP and subsequent microarray analysis. The integrity of the RNAs was then examined using an Agilent 2100 Bioanalyzer,

Table II. Top 20 antisense transcripts differentially regulated between blood cells from patients with colorectal cancer and healthy volunteers.

Accession number	Gene symbol	Gene name	Fold-change
NM_012080	HDHD1	Haloacid dehalogenase-like hydrolase domain-containing 1	5.68
NM_005824	LRRC17	Leucine-rich repeat containing 17	4.38
XR_016125	LOC642337	Similar to hCG1648021	3.40
NM_016630	SPG21	Spastic paraplegia 21	2.71
XM_001132492	LOC732276	Hypothetical protein LOC732276	2.54
NM_175611	GRIK1	Glutamate receptor, ionotropic, kainate 1	2.54
NM_003290	TPM4	Tropomyosin 4	2.40
NM_006516	SLC2A1	Solute carrier family 2, member 1	2.39
NM_015317	PUM2	Pumilio homolog 2	2.36
NM_024494	WNT2B	Wingless-type MMTV integration site family, member 2B	-2.35
NM_025140	CCDC92	Coiled-coil domain-containing 92	2.31
NM_001037738	NPM1	Nucleophosmin 1	2.31
NM_024860	SETD6	SET domain-containing 6	2.27
NM_020179	SMCO4	Homo sapiens chromosome 11 open reading frame 75 (C11orf75)	2.25
XR_016982	LOC645280	Hypothetical LOC645280	2.21
NM_025225	PNPLA3	Homo sapiens patatin-like phospholipase domain-containing 3	2.20
NM_021975	RELA	Homo sapiens v-rel avian reticuloendotheliosis viral oncogene homolog A	2.03
XM_926307	LOC642927	Similar to COLLAGEN family member (col-36)	2.02
NM_172249	CSF2RA	Homo sapiens colony stimulating factor 2 receptor, alpha, low-affinity	2.02
NM_005206	CPK	Homo sapiens v-crK avian sarcoma virus CT10 oncogene homolog	2.00

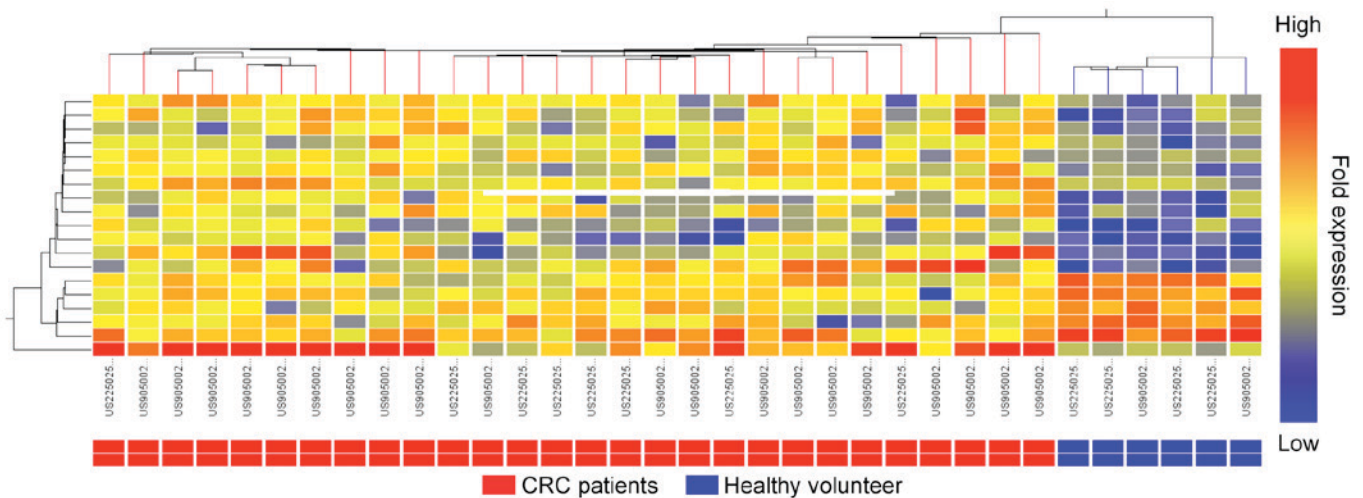


Figure 1. Hierarchical cluster analysis of the patients with CRC and the healthy volunteers using the top 20 antisense transcripts presented in Table II. Heat maps depict color-coded expression levels (the color gradation from red to blue indicates high to low expression levels). CRC, colorectal cancer.

revealing that the Rin values of the RNA samples ranged from 5.9 to 9.2. As the random priming method was utilized for the synthesis of Cy3-labeled cDNAs from the RNA samples, the lower Rin values were considered not to affect the quality of the cDNAs for analysis.

Microarray analysis. Cy3-labeled cDNAs were synthesized from the extracted RNA and subjected to microarray analysis. A total of 40 transcripts were identified as differentially

expressed with a magnitude of >2 -fold ($P < 0.05$) between the CRC blood cells and non-cancerous blood cells; of these transcripts, 20 were sense sequences and 20 were antisense sequences (Table II). According to the cluster analysis, CRC and non-cancerous blood samples were revealed to form separate clusters for the antisense transcripts, but not to form separate clusters for the sense transcripts (Fig. 1; the clustering data for the sense transcripts are not presented).

Table III. Top 39 antisense transcripts differentially regulated between blood cells from patients with stage I/II colorectal cancer and healthy volunteers.

Accession number	Gene symbol	Gene name	Fold-change
XM_001132487.1ea	LOC732271	Hypothetical protein LOC732271	6.24
NM_005824.1ea	LRRC17	Leucine-rich repeat-containing 17	4.50
NM_012080.3ea	HDHD1	Haloacid dehalogenase-like hydrolase domain-containing 1	3.57
NM_024422.2ia	DSC2	Desmocollin 2	2.94
NM_174913.1ia	NOP9	NOP9 nucleolar protein	2.92
NM_016630.3ia	SPG21	Spastic paraplegia 21	2.71
NM_024494.1ia	WNT2B	Wingless-type MMTV integration site family, member 2B	2.61
NM_175611.2ea	GRIK1	Glutamate receptor, ionotropic, kainate 1	2.54
NM_014578.2ia	RHOD	Ras homolog family member D	2.54
NM_006296.3ia	VRK2	Vaccinia-related kinase 2	2.47
NM_139284.1ia	LGI4	Leucine-rich repeat LGI family	2.44
NM_001315.1ia	MAPK14	Mitogen-activated protein kinase 14	2.40
XR_016125.1ea	LOC642337	Similar to hCG1648021	2.39
NM_005618.2ia	DLL1	Felta-like 1 (Drosophila)	2.37
XM_001132492.1ea	LOC732276	Hypothetical protein LOC732276	2.36
NM_025225.2ia	PNPLA3	Patatin-like phospholipase domain-containing 3	2.31
NM_171846.1ea	LACTB	Lactamase, β	2.31
NR_002162.1ea	ATP5EP2	Synthase, H ⁺ transporting, mitochondrial F1 complex, epsilon subunit pseudogene 2	2.29
XM_001125928.1ea	LOC283804	Similar to testicular metalloprotease-like, disintegrin-like, Cysteine-rich protein Iva	2.25
XM_001129971.1ea	LOC729333	Hypothetical protein LOC729333	2.23
NM_212554.2ia	METTL10	Methyltransferase-like 10	2.22
NM_006516.1ia	SLC2A1	Solute carrier family 2, member 1	2.20
NM_015317.1ia	PUM2	Pumilio homolog 2	2.18
XM_293121.4ea	C20orf66	Chromosome 20 open reading frame 66	2.15
NM_003290.1ia	TPM4	Tropomyosin 4	2.12
XR_016982.1ea	LOC645280	Hypothetical LOC645280	2.12
XR_018204.1ea	LOC647757	Similar to tetratricopeptide repeat protein 4 (TPR repeat protein 4)	2.09
NM_006708.1ia	GLO1	Glyoxalase I	2.08
NM_020179.1ia	SMCO4	Single-pass membrane protein with coiled-coil domains 4	2.06
NM_001010898.1ia	SLC6A17	Solute carrier family 6 (neutral amino acid transporter), member 17	2.06
NM_004423.3ia	DVL3	Dishevelled segment polarity protein 3	2.05
NM_001093.2ia	ACACB	Acetyl-CoA carboxylase β	2.04
NM_005751.3ia	AKAP9	A kinase (PRKA) anchor protein 9	2.04
NM_025140.1ia	CCDC92	Coiled-coil domain-containing 92	2.04
NM_017440.2ia	MDM1	Mdm1 nuclear protein homolog (mouse)	2.04
NM_000637.2ia	GSR	Glutathione reductase	2.03
NM_080588.1ia	PTPN7	Protein tyrosine phosphatase, non-receptor type 7	2.02
NM_024860.1ia	SETD6	SET domain-containing 6	2.01
NM_005206.3ia	CRK	V-crk avian sarcoma virus CT10 oncogene homolog	2.01

PCA for 28 patients with CRC using 20 antisense transcripts. As presented in Fig. 2, PCA analysis revealed that the CRC and non-cancerous blood samples were well separated with the first principal component; its contribution rate was ~43.5%. The contribution rates of the second and third principal components were calculated to be ~7.7% and ~6.4%, respectively. These results indicate that the CRC and non-cancerous

blood samples were effectively separated with only the first component.

Hierarchical cluster analysis of patients with stage I or II CRC. The results of the microarray with RNA samples from patients with stage I and II CRC and controls revealed a total of 72 transcripts that were significantly differentially expressed

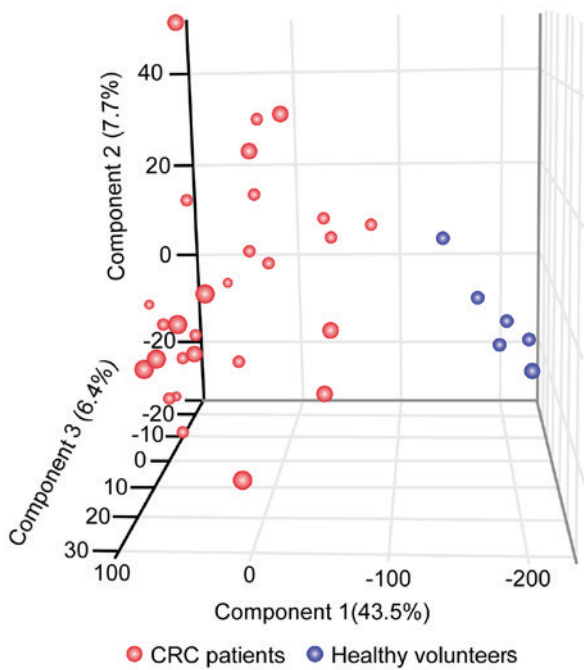


Figure 2. Principal component analysis for 28 patients with CRC and 6 healthy volunteers using the 20 antisense transcripts presented in Table II. The data are representative of the top three components; the contribution ratios of components 1, 2 and 3 were 43.5, 7.7 and 6.4%, respectively. CRC, colorectal cancer.

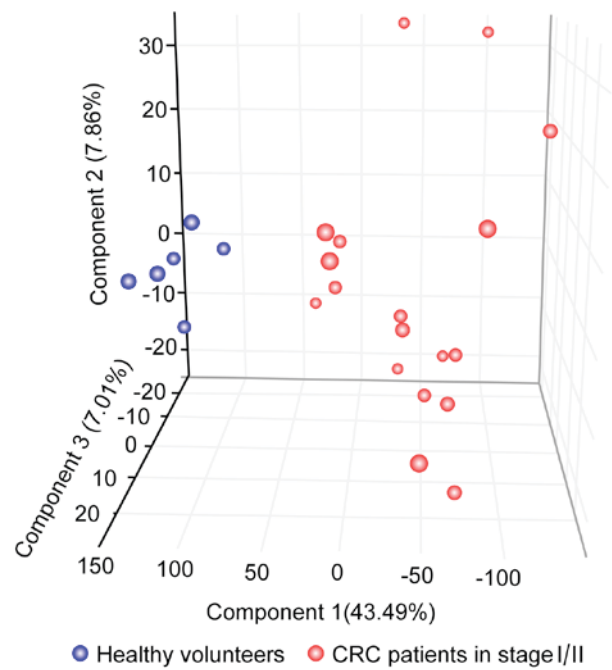


Figure 4. Principal component analysis for 18 patients with stage I or II CRC and 6 healthy volunteers using the 39 antisense transcripts presented in Table III. The data are representative of the top 3 components; the contribution ratios of components 1, 2 and 3 were 43.5, 7.9 and 7.0%, respectively. CRC, colorectal cancer.

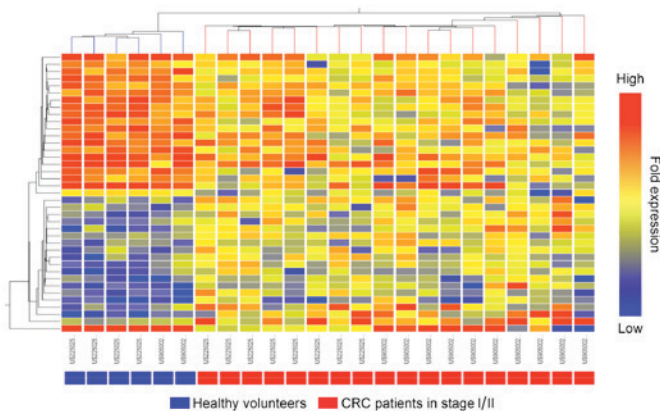


Figure 3. Hierarchical cluster analysis of patients with stage I or II CRC and healthy volunteers, using the top 39 antisense transcripts presented in Table III. Heat maps depict color-coded expression levels (the color gradation from red to blue indicates high to low expression levels). CRC, colorectal cancer.

with a magnitude of >2 -fold ($P < 0.01$) between the blood cells of healthy volunteers and of patients with CRC. A total of 33 transcripts were sense sequences and 39 were antisense sequences (Table III). When the blood samples from patients with stage I or II CRC and healthy volunteers were subjected to cluster analysis for the aforementioned sense and antisense transcripts, the patients were observed to form respective clusters only with antisense transcripts (Fig. 3; the clustering data for the sense transcripts are not presented).

PCA with stage I or II CRC using 39 antisense transcripts. The 39 antisense transcripts were further examined using PCA

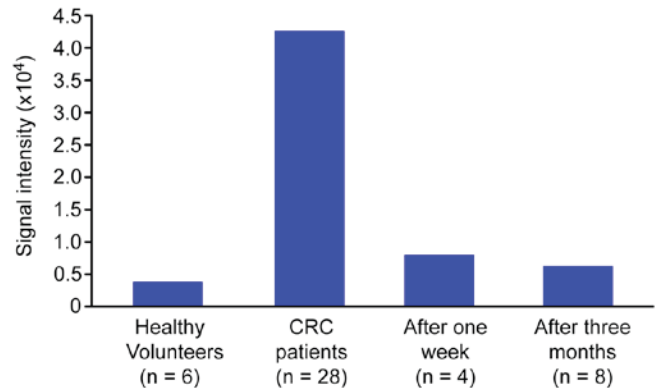


Figure 5. Comparison of haloacid dehalogenase-like hydrolase domain-containing 1 antisense transcript expression levels in the blood cell samples of healthy volunteers and patients with CRC prior to surgery and at one week and three months after surgery. CRC, colorectal cancer.

analysis. As presented in Fig. 4, CRC and non-cancerous RNA samples were revealed to be well separated with regard to the first principal component; its contribution rate was $\sim 43.5\%$. The contribution rates of the second and third principal components were calculated to be $\sim 7.9\%$ and $\sim 7.0\%$, respectively. These results indicate that the patients with stage I or II CRC were effectively separated from healthy volunteers with the first component.

Comparison of HDHD1 antisense transcript expression levels. The antisense transcript of haloacid dehalogenase-like hydrolase 1 domain-containing (HDHD1), which was the highest and third highest differentially expressed transcript in the earlier analyses (Tables II and III, respectively), was

selected for evaluation at various time points following the surgical resection of tumor tissues from patients with CRC. The expression levels of the HDHD1 antisense transcript at 1 week and at 3 months post-surgery were decreased approximately to the expression levels observed in healthy volunteers (Fig. 5). The results suggest that certain antisense transcripts, including HDHD1, may serve as potential biomarkers of CRC diagnosis and prognosis.

Discussion

Previous studies have identified the potential involvement of antisense RNA expression in the development of colorectal and hepatic cancers by examining RNA expression patterns in cancerous and healthy tissues (14,15). However, the functions and underlying mechanisms of antisense transcripts in colorectal and hepatic cancers have yet to be elucidated. The present study examined the association between the expression levels of certain RNA transcripts in blood cells and the occurrence of CRC, and the subsequent changes in the transcript amount following the removal of cancerous tissues in patients. The examinations revealed that antisense transcripts of up to 39 genes demonstrated a clear association between their expression levels in blood cells and the occurrence of CRC; following tumor resection in patients with CRC, the expression levels of the HDHD1 antisense transcript were decreased to approximately the levels observed in healthy volunteers, suggesting that these antisense transcripts are involved in the generation and maintenance of CRC. Furthermore, the antisense transcripts may serve as diagnostic markers for CRC occurrence, and certain antisense transcripts, including HDHD1, may be potential prognostic markers for CRC.

The early detection of CRC significantly improves patient prognosis and is essential in reducing CRC-associated mortality (19). Patients with CRC often present with an advanced stage disease and concomitant poor prognosis (1). The best known serum biomarkers, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), are not recommended for clinical screening due to limited specificity and sensitivity (20). A number of circulating proteins have previously been indicated to be diagnostically useful; however, none of these proteins has individually demonstrated sufficient sensitivity or specificity to be used in clinical practice (20).

In our previous study, the expression levels of certain antisense transcripts in CRC tissues were revealed to significantly differ from their corresponding normal tissues (14), indicating that those specific antisense transcripts may be involved in the generation and maintenance of CRC tumor tissues. The antisense transcripts in CRC tissues are distinct from those identified in the present study of patients with CRC. Further studies are required to determine the functional association between the antisense transcripts revealed in our previous study and those in the present study.

Previous studies have indicated that certain antisense transcripts are involved in mRNA stabilization (21), the suppression of mRNA synthesis (22,23), miRNA functions (24) and the promotion of protein synthesis (25).

Therefore, antisense transcripts have various functions, each of which may be specific to the respective antisense transcript species. Although the functions of non-coding RNA, antisense RNA and miRNA in tumorigenesis require further study, it is possible that the various antisense transcripts demonstrated in the present study may serve as potential biomarkers for CRC diagnosis and prognosis. In the future, the mechanisms underlying the differences in expression levels of the antisense transcripts should be investigated extensively to understand their involvement in CRC generation.

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