

# Differential expression profiles of sense and antisense transcripts between HCV-associated hepatocellular carcinoma and corresponding non-cancerous liver tissue

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**Abstract.** Recent studies have demonstrated that natural antisense transcripts, which are complementary sequences to messenger RNA, have important cellular functions such as the stabilization and silencing of mRNA. However, the possible contribution of antisense transcripts in hepatocellular carcinoma (HCC) development has not been described. Therefore, we simultaneously investigated the sense and antisense transcripts of HCC and non-cancerous tissues to explore the possible contribution of antisense transcripts to HCC progression. RNA was prepared from 15 HCV-associated HCCs and from 6 corresponding non-cancerous tissues and was subjected to expression profile analysis of sense and antisense transcripts using a human custom microarray. Differential expression of 161 sense and 25 antisense transcripts was observed with more than 2-fold between HCC and non-cancerous tissue ( $p < 0.001$ ). The expression of the sense and antisense transcripts was used to cluster cancer and non-cancerous tissues, and the cancer and non-cancerous tissues were found to be clearly separated into different clusters. Additionally, the sense and antisense expression profiles were analyzed with regard to HCC differentiation ( $p < 0.001$ ), resulting in 71 sense and 43 antisense transcripts. These unique transcripts did not overlap with those found in the discrimination of HCC from non-cancerous tissues. When the HCC tissues were clustered by transcript expression, the antisense transcripts resulted in clustering of HCC that was consistent with grouping based on histology. These findings

strongly indicate that the antisense transcripts together with the sense transcripts are involved in liver tumorigenesis.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third cause of cancer-related deaths (1). However, only the 30% of patients who are at a very early stage of HCC receive benefits from curative therapies (2) and achieve 5-year survival rates of 50-70% (3); the remaining 70% of patients receive palliative treatments. Therefore, the detection of HCC at very early stages is a prerequisite for higher survival rates. In addition, the degree of differentiation of HCC is relevant to patient prognosis after curative treatments (4). In order to achieve early detection and a more accurate prognosis, HCC has been investigated in a variety of aspects such as histology, cytology, and biochemistry.

The advent of microarray analysis and mass-scale sequencing technologies has enabled the gene-expression profiling of cells, which has led us to compare the profiles of HCC with those of non-cancerous liver tissues. Previously, mRNA (5) and microRNA (6-8) have been reported to be biomarkers distinguishing HCC from non-cancerous liver tissues (9,10). Despite such progress, the characterization of HCC and the prognosis of post-curative treatments using these biomarkers are still insufficient for practical use.

Recently, non-coding RNA such as microRNA, Piwi-interacting RNA, small nuclear RNA, and natural antisense transcripts have received attention due to their regulatory effects on genes (11-14). Antisense transcripts, a non-coding RNA species complementary to other intracellular RNA (15,16), have been systematically identified in mammalian species (17); it has been indicated by widespread transcriptome analysis that up to 70% of human transcripts have antisense partners (18). Recent studies have reported that antisense transcripts are associated with a variety of gene regulation in eukaryotes (19,20). For example, in Alzheimer's disease, BACE1 antisense transcripts form an RNA duplex with BACE1 mRNA and increase the stability of BACE1 mRNA (19). In leukemia, CDKN2B (also

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called p15) antisense transcripts may be a trigger for heterochromatin formation in tumor suppressor genes through the inverse relation between p15 sense and antisense expression (20).

However, a comprehensive antisense transcript analysis in human HCC samples has not yet been reported. In this study, we investigated the sense and antisense transcript profiles in HCC and non-cancerous liver tissues using a custom microarray in order to determine the possible involvement of these transcripts in liver tumorigenesis.

## Patients and methods

**Patients and samples.** HCCs were resected from HCV-positive patients at the University of Tsukuba Hospital, frozen in liquid nitrogen, and stored at -80°C. The HCC specimens were collected from 15 patients who underwent surgical resection from August, 2006 through March, 2009. The study was approved by the hospital ethics committee and informed consent was obtained from all patients. Prior to RNA preparation, tissues were subjected to histological examination to select HCCs and non-cancerous sections. As listed in Table I, the sections were obtained from 15 HCCs and 6 non-cancerous tissues as samples for microarray analysis.

**Total RNA extraction.** Total RNA was isolated from frozen samples using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentrations were determined by measuring the absorbance at 260/280 nm on the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's instructions. The integrity of the total RNA was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and an RNA 6000 Nano LabChip kit (Agilent Technologies).

**Probe design of the custom microarray.** One of the steps in microarray gene expression analysis is to use *in situ* hybridization to investigate gene expression sites in tissues. The probe size for *in situ* hybridization should be approximately 120 nucleotides (nt) to obtain a satisfactory hybridization signal/noise ratio. Since the copy numbers of gene transcripts have been shown to vary depending on the gene region (21), probe sequences for microarray and for *in situ* hybridization should be selected in the same gene region in order to interpret the results of microarray in combination with *in situ* hybridization. Therefore, 120 nt sequences were first selected from human ORF sequences (Build35) for use as a probe for *in situ* hybridization (Genetyx, Tokyo, Japan). The selected sequences were confirmed to be unique in the human genomic sequence by BLAST analysis, and were then submitted to the Agilent server (Agilent Technologies) to design 60 nt sequences for use as microarray probes. The sense and antisense sequences of 60 nt were arranged in an Agilent 44 K x 4 system (20882 ORFs: Agilent eArray Design ID 19052 produced by Tsukuba Gene Technology Laboratories, Tsukuba, Japan) (Agilent Technologies).

**Microarray analysis.** Cyanine 3 (Cy3)-labeled cDNA was synthesized from 10 µg total RNA of HCC and non-cancerous samples using a LabelStar Array kit (Qiagen, Valencia, CA),

Cy3-dUTP (GE Healthcare, Fairfield, CT), and a random nonamer primer. The labeled cDNA was hybridized with probe sequences on an Agilent 44 K x 4 human sense and antisense custom microarray slide (22) in a hybridization solution prepared with In Situ Hybridization kit Plus (Agilent Technologies), following the manufacturer's instructions. The Cy3 fluorescence signal images on the slides were obtained by a DNA microarray scanner (Agilent Technologies) and processed using the Feature Extraction version 8.1 software based on instruction from Agilent Technologies. The expression data thus obtained were processed using Gene Spring GX version 11.5 software (Agilent Technologies) to perform a log transformation and normalization to the 75 percentile of all values on the respective microarrays, followed by normalization of the median expression level of all samples. Additionally, the normalized gene expression data were filtered on flags, and only those genes classified as either flag-Present or flag-Marginal in >70% of all samples, were allowed to pass the filter.

The expression profiles of the samples were compared using unpaired t-tests (with Benjamini-Hochberg FDR correction for unequal variances) as described in the Results section. Two-dimensional hierarchical clustering based on Euclidean distance measures was performed using Ward's method. Results are visualized with the help of heat maps and dendrograms. The heat maps show color-coded expression levels; the color gradation from red to blue indicates the expression levels from high to low. Sample trees are drawn horizontally and gene trees were drawn vertically. To identify genes with statistically significant differences in tumors with varying degrees of differentiation, a one-way ANOVA with a p-value cutoff of 0.001 was performed. To characterize the trends of multigene expression, we used principal component analysis (PCA).

## Results

**Identification of sense and antisense transcripts differentially expressed between HCV-associated HCC and non-cancerous liver tissue.** In order to determine the sense and antisense transcripts that are differentially expressed between HCC and non-cancerous liver tissue, total RNA was prepared as described in Patients and methods. To reduce background differences, samples were obtained only from HCV-positive patients. All total RNAs were shown to have 260/280 nm absorbance ratios of 1.8-2.0 and integrity values (RIN) ranging from 7.9-9.9. Based on the instruction for microarray analysis (Agilent Technologies), the quality of the RNA was determined to be appropriate for microarray analysis.

The total RNA from 15 HCC tissues and 6 corresponding non-cancerous liver tissues (Table I) was subjected to microarray analysis as described in Patients and methods. It was determined that 186 transcripts were differentially expressed between HCC and non-cancerous liver tissues with a magnitude of more than 2-fold, based on an unpaired t-test ( $p < 0.001$ ); 161 transcripts were sense sequences and the remaining 25 transcripts were antisense sequences (Table II). Of the 161 sense transcripts, 138 were found to be up-regulated, and the remaining 23 were down-regulated. Furthermore, of the 25 antisense transcripts, 18 were found to be up-regulated, and the remaining 7 were down-regulated. Upon examination for the presence of common genes, the sense transcript (mRNA) and antisense transcript of

Table I. Characteristics of patients, as used for microarray analysis.

Sample no.	Age	Gender	Histology	AFP	PIVKA-II	Fibrosis score(F)	Activity score(A)	Normal tissue
35	79	M	Mod	71	2291	3	2	-
36	74	M	Mod	10	23	3	2	Yes
50	75	M	Well	8	33	4	1-2	-
56	70	M	Well	33	14	3	2	Yes
59	75	F	Mod	263	37	3	2	-
62	71	M	Well	39	184	3	2	-
64	75	M	Well	41	11	4	2	Yes
66	49	M	Well	11	97	4	2	-
69	60	M	Poor	2	35	2	1	-
73	69	F	Mod	762	68	4	2	Yes
75	75	M	Mod	6	253	2	2	-
77	54	M	Poor	5417	933	-	-	-
79	57	M	Poor	258	149	4	2	Yes
96	52	M	Mod	91	95	3	1	Yes
98	71	M	Mod	46	32649	2	1	-

F, fibrosis; A, activity; Mod, moderate.

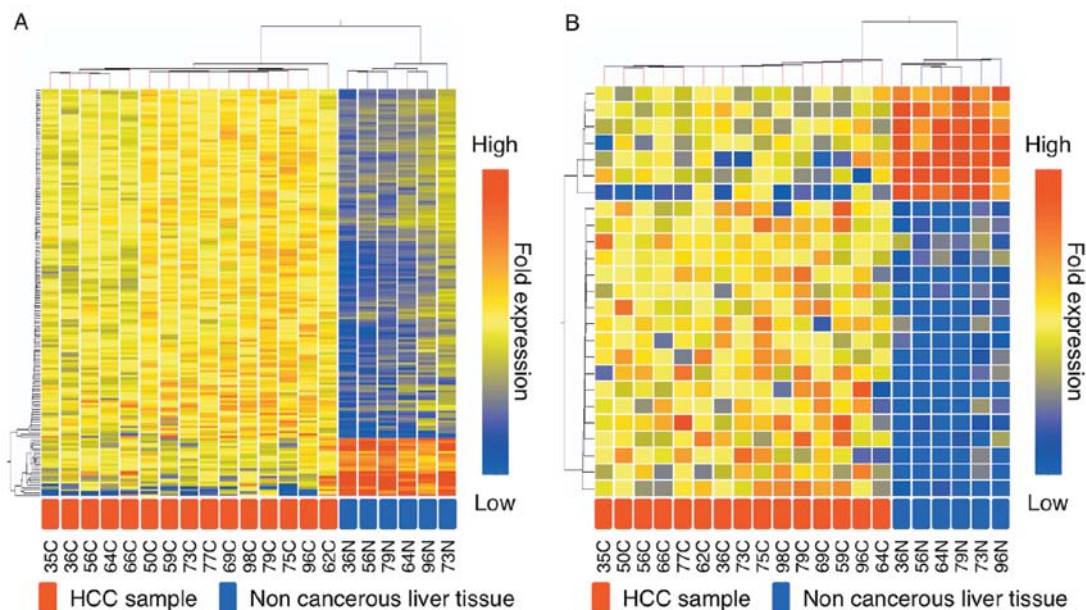


Figure 1. (A) A hierarchical cluster analysis using 161 up- and down-regulated sense transcripts of HCC and non-cancerous liver tissue samples. The heat maps show color-coded expression levels (the color gradation from red to blue indicates the expression levels from high to low). A blue box at the bottom of the image indicates non-cancerous samples; a red box indicates HCC samples. (B) A hierarchical cluster analysis using 25 up- and down-regulated antisense transcripts of HCC and non-cancerous liver tissue samples.

metadherin (MTDH) were both found to be up-regulated in HCCs.

Some of the 161 sense transcripts have been already demonstrated to be candidates for markers of HCC (23-30). This fact provided support for the assumption that the microarray results accurately reflect gene expression of HCC and non-cancerous tissues. In addition, analysis of the custom microarray for colorectal cancer produced results that were consistent with

those of quantitative RT-PCR (22). Therefore, without verification of the array results by quantitative RT-PCR, we proceeded to the clustering analysis of the 161 sense and 25 antisense transcripts. HCC samples and non-cancerous tissue samples were separately clustered for sense transcripts as well as antisense transcripts as shown in Fig. 1A and B. These results demonstrated that expression profiles of sense and antisense transcripts varied between HCC and non-cancerous tissues.

Table II. Twenty-five natural antisense transcripts differentially regulated between HCC and non-cancerous liver tissue.

Accession no.	Gene symbol	Gene name	Fold change	Regulation
NM001018136.1	NME1-NME2	NM23-LV	4.0762205	Up
NM005896.2	IDH1	Isocitrate dehydrogenase 1 (NADP <sup>+</sup> ), soluble	3.9515014	Up
NM000989.2	RPL30	Ribosomal protein L30	3.9422822	Up
NM016032.2	ZDHHC9	Zinc finger, DHHC-type containing 9	3.8781722	Up
NM024671.2	ZNF768	Zinc finger protein 768	3.800295	Up
NM198880.1	QRICH1	Glutamine-rich 1	3.7468915	Up
NM016645.2	NGRN	Neugrin, neurite outgrowth associated	3.6181226	Up
NM006808.2	SEC61B	Sec61 $\beta$ subunit	3.568331	Up
NM015289.2	VPS39	Vacuolar protein sorting 39 (yeast)	3.4819472	Up
NM005005.1	NDUFB9	NADH dehydrogenase (ubiquinone), 9, 22 kDa 1 $\beta$ subcomplex	3.4809504	Up
NM203298.1	CHCHD1	Coiled-coil-helix-coiled-coil-helix domain containing 1	3.3855672	Up
NM006070.4	TFG	TRK-fused gene	3.2429345	Up
NM181528.2	NAT5	N-acetyltransferase 5	3.0416958	Up
NM001007027.2	ALG8	Asparagine-linked glycosylation 8 homolog ( <i>S. cerevisiae</i> , $\alpha$ -1,3-glucosyltransferase)	2.9185905	Up
NM006694.2	JTB	Jumping translocation breakpoint	2.889171	Up
XM001133555.1	LOC729130	Similar to phosphodiesterase 4D interacting protein isoform 1	2.5856044	Up
NM001039712.1	DEDD	Death effector domain containing	2.037344	Up
NM178812.2	MTDH	Metadherin	2.027995	Up
NM012174.1	FBXW8	F-box and WD-40 domain protein 8	5.336158	Down
NM133507.2	DCN	Decorin	3.7978623	Down
NM000598.4	IGFBP3	Insulin-like growth factor binding protein 3	3.5457635	Down
NM001781.1	CD69	CD69 molecule	3.4615583	Down
NM171825.1	CAMK2A	Calcium/calmodulin-dependent protein kinase (CaM kinase) II $\alpha$	2.8482397	Down
XR018502.1	LOC644202	Similar to 60S ribosomal protein L12	2.426251	Down
NM015658.1	NOC2L	Nucleolar complex associated 2 homolog ( <i>S. cerevisiae</i> )	2.349828	Down

The sense and the antisense transcripts thus identified were further investigated using PCA analysis. As shown in Fig. 2A and B, HCC and non-cancerous tissues were found to be well separated with the first principal component (PC1); contribution rates were 86.4 and 79.1% for sense and antisense transcripts, respectively. The contribution rates of the second principal component (PC2) were calculated to be 3.0 and 4.2% for sense and antisense transcripts, respectively. These findings together indicate that the PC1 was sufficient for the separate clustering of HCC and non-cancerous tissues.

When the constitution of PC1 and PC2 for sense transcripts was examined, respective transcripts were found to contribute to PC1 at nearly equal levels and to PC2 at various levels (data not shown). Similarly, with regards to the antisense transcripts, respective transcripts were found to contribute to PC1 at nearly equal levels and to PC2 at various levels (data not shown).

*Identification of sense and antisense transcripts differentially expressed with respect to differentiation stages of HCCs.* The histological differentiation levels of HCC have been demonstrated

to be strongly related to prognosis of HCC (4). Therefore, we then examined whether the expression profiles distinguished the histological differentiation of HCC. The results of the microarray analysis were subjected to statistical analysis in terms of histological differentiation of HCCs (5 were well differentiated, 7 were moderately differentiated, and 3 were poorly differentiated) using the one-way ANOVA, followed by the Tukey HSD post-hoc test ( $P < 0.001$ ). The analysis allowed us to select 71 sense and 43 antisense transcripts. Of these transcripts, only one gene, a corticotropin releasing hormone binding protein (CRHBP), was present in both sense and antisense transcripts.

First, we performed clustering of the 15 HCCs using the 71 sense transcripts. As a result of the clustering shown in Fig. 3A, HCCs with the same differentiation stage except 36C HCC were clustered into the same group. The 36C HCC, which was shown to be moderately differentiated, was clustered with the well differentiated HCCs. The expression profiles of the 71 sense transcripts in the 15 HCCs were further investigated with PCA analysis, revealing that the HCCs were separated into groups depending on differentiation stages when PC1 (contribution

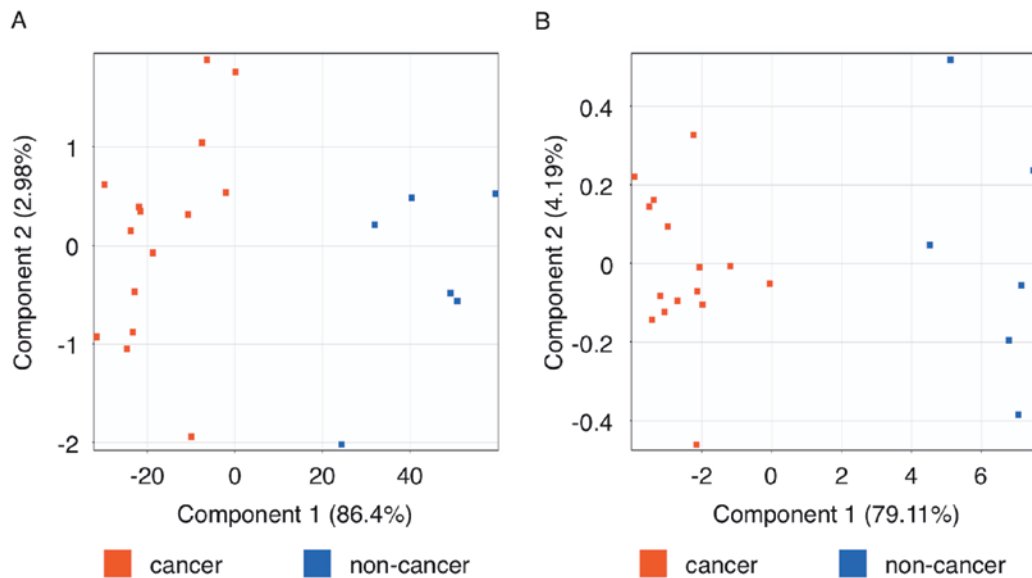


Figure 2. (A) A graphical presentation of principal component analysis (PCA) for 15 HCC samples and 6 corresponding non-cancerous samples using 161 sense transcripts based on PC1 (contribution ratio: 86.4%) and PC2 (contribution ratio: 3.0%). A blue box indicates non-cancerous samples; a red box indicates HCC samples. (B) A graphical presentation of principal component analysis (PCA) for 15 HCC samples and 6 corresponding non-cancerous samples using 25 antisense transcripts based on PC1 (contribution ratio: 79.1%) and PC2 (contribution ratio: 4.2%). A blue box indicates non-cancerous samples; a red box indicates HCC samples.

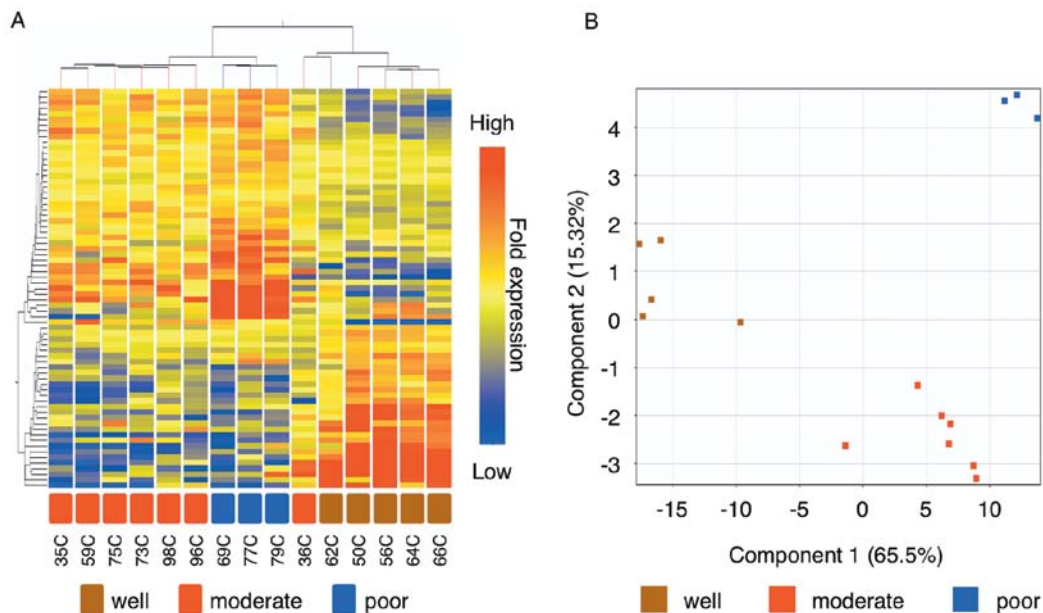


Figure 3. (A) A hierarchical cluster analysis using 71 sense transcripts of HCC and non-cancerous liver tissue samples. The heat maps show color-coded expression levels (the color gradation from red to blue indicates the expression levels from high to low). A red box at the bottom of the image indicates moderate differentiation; a blue box indicates poor differentiation, and a brown box indicates well differentiated sample. (B) A graphical presentation of principal component analysis (PCA) for 15 HCC samples using 71 sense transcripts based on PC1 (contribution ratio: 65.5%) and PC2 (contribution ratio: 15.3%). A brown box indicates well differentiated sample; a red box indicates moderate differentiation, and a blue box indicates poor differentiation.

rate: 65.5%) and PC2 (contribution rate: 15.3%) were used for scatter plot (Fig. 3B). For PC1, 58 transcripts were found to contribute to the component at nearly equal levels; the remaining 13 contributed to the component less than the 58 transcripts (data not shown). For PC2, contribution rates of the 71 transcripts were variable (data not shown).

Next, we performed clustering of the HCCs using the 43 antisense transcripts, revealing that HCCs with the same

differentiation stages were clustered into the same group (Fig. 4A). As in the sense transcripts, the expression profiles of the 43 antisense transcripts were investigated with PCA analysis, which revealed the following: the HCCs were separated into groups depending on differentiation stage, when PC1 (contribution rate: 60.3%) and PC2 (contribution rate: 17.6%) were used for a scatter plot (Fig. 4B). For PC1, 32 transcripts were found to contribute to the component at nearly equal levels,



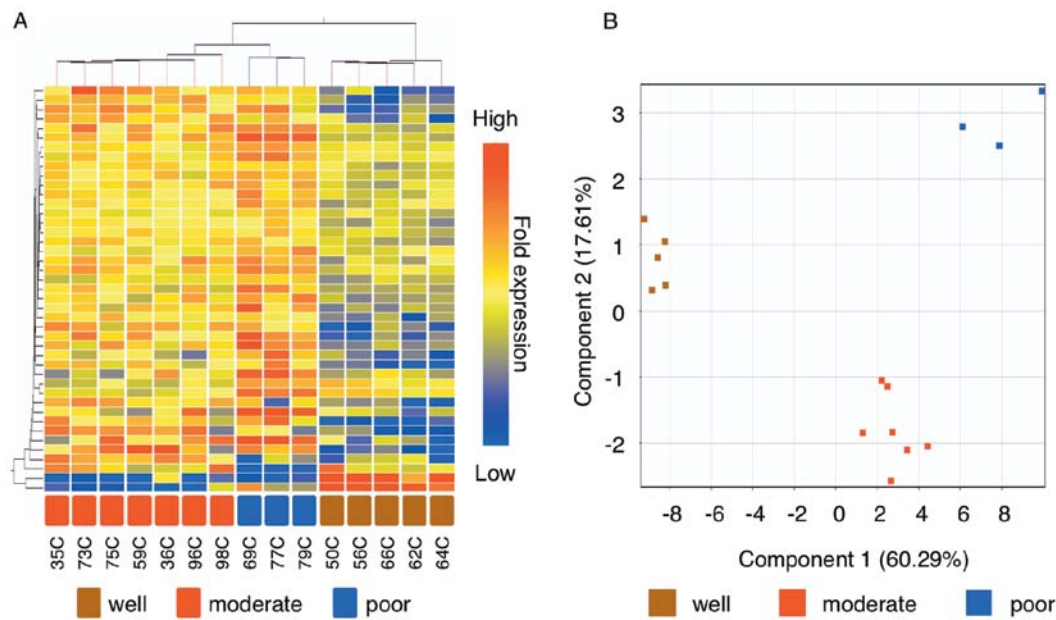


Figure 4. (A) A hierarchical cluster analysis using selected 43 antisense transcripts of HCC and non-cancerous liver tissue samples. The heat maps show color-coded expression levels (the color gradation from red to blue indicates the expression levels from high to low). A red box at the bottom of the image indicates moderate differentiation; a blue box indicates poor differentiation, and a brown box indicates a well differentiated sample. (B) A graphical presentation of principal component analysis (PCA) for 15 HCC samples using 43 antisense transcripts based on PC1 (contribution ratio: 60.3%) and PC2 (contribution ratio: 17.6%). A brown box indicates a well differentiated sample; a red box indicates moderate differentiation, and a blue box indicates poor differentiation.

and the remaining 11 contributed to the component less than the 32 transcripts (data not shown). For PC2, contribution rates of the 43 transcripts were variable (data not shown).

The findings obtained in the present study are summarized as follows: The HCCs were clearly distinguished from non-cancerous liver tissues by clustering and PCA analyses using the expression profiles of the 161 sense and the 25 antisense transcripts. Of the transcripts, only one gene, MTDH, was altered in the sense as well as antisense transcripts. In addition, the grouping of HCCs based on their differentiation stages determined by histological examination was consistent with that based on the clustering and PCA analyses using the expression profiles of the 43 antisense transcripts, and essentially with that using the 71 sense transcripts. In these transcripts, only one gene, CRHBP, was changed in the sense as well as antisense transcripts. When the transcripts selected for discrimination of cancer from normal tissues were compared with those for HCCs differentiation, no transcripts were found to be overlapping.

## Discussion

Many studies in the past decade have shown that epigenetic changes can play an important role in hepatocarcinogenesis (31-33). Several studies have focused on the expression profiling of HCC using microarrays, and discovered sense transcripts (5,28,34-37) and miRNAs (6-10) distinguish cancerous from non-cancerous tissues. These studies revealed that there are different expression patterns between cancerous and non-cancerous tissues. On the other hand, some studies have reported that there are NATs expression change in Alzheimer's disease (19), leukemia (20), CRC (22), breast cancer (38). In the present study, we simultaneously investigated the profiles of sense and antisense transcripts of HCC and non-cancerous liver tissues to explore the possible contribution of antisense transcripts

to HCC progression. To our knowledge, this is the first study showing the comprehensive analysis of NATs in human HCC and non-cancerous tissues in order to seek specific biomarkers for HCC and to clarify the mechanism of HCC development.

Gene expression profiles of HCC on mRNA and miRNA have been reported with significantly different expression levels between cancerous and non-cancerous tissues (5,28,34-37). We investigated the antisense transcripts that distinguish cancerous tissues from non-cancerous tissues in HCV-associated liver. Our result indicated that it is possible to detect cancer-specific expression patterns using antisense transcripts. In this study, we also investigated the profile of sense transcripts, and expression changes in the gene, DPT (39), CXCL14 (40), HAMP (41), MAGE1A (42) were consistent with results of previous studies. In contrast to genes discussed above, some genes that were reported previously were not recognized in the present study. This discrepancy may be due to different sample size and different infected virus types of the patients.

Although the function of antisense transcripts is not yet well understood, recent studies have shown that antisense transcripts play a role to stabilize mRNA (43), and/or silencing mRNA (20). Moreover, Faghihi *et al* reported antisense transcript-mediated inhibition of miRNA function (44). In the present study, we investigated the expression levels of sense-antisense pairs in cancerous and non-cancerous tissue. We observed both positive and negative correlations of sense-antisense expression levels ( $p < 0.05$ , fold change  $> 2.0$ ), and  $> 75\%$  (data not shown) were positive correlation. This result was consistent with the results of Grigoriadis *et al* (38) reported in breast tumors and suggests that antisense transcripts both stabilize and silence of mRNA function.

Multistep tumorigenesis was reported in colorectal cancer (45). HCC is also characterized by multistep process of

tumor progression by histopathological analyses (46,47). Nakanishi *et al* reported that tumor differentiation is one of the risk factors for poor prognosis (4). Midorikawa *et al* reported several genes are likely to be associated with dedifferentiation of HCC (48). We analyzed gene expression profiles and found differentially expressed antisense transcripts with strong relation to the histological differentiations. In clustering analysis of this study, samples were separately clustered into two groups i.e., well differentiated cluster, and moderately plus poorly differentiated cluster. Patient no. 36, whose cancerous tissue was histologically classified as moderately differentiated HCC, was clustered with the well differentiated HCCs by sense transcripts (Fig. 3A), but this patient was clustered with moderately differentiated HCCs according to antisense transcripts (Fig. 4A). We re-inspected the histological findings of the patient, but diagnosis of moderately differentiated carcinoma was confirmed. These findings combined with the histological examination indicated that the antisense transcripts are a more suitable parameter for the determination of HCC profile than the sense transcripts. However, since there is still a possibility that, although the patient was grouped into the moderately differentiated cluster, the character of the patient might be different from the other moderately differentiated HCCs, the patient should be carefully followed after treatment. This result described above indicates that analysis of HCC by antisense transcripts would stratify patients more accurate than by sense transcripts.

In the present study, not only sense transcripts but also antisense transcripts were demonstrated to be involved in liver tumorigenesis and de-differentiation of HCC cells. These results indicate that expression profile of antisense transcripts contribute to prediction of prognosis and recurrence types, and to decisions for treatment strategy such as surgery, chemotherapy, and transcatheter arterial embolization. As for the selection of biomarkers for clinical use, a large number of HCC samples should be examined to select reliable biomarkers and analyze the relationship with prognosis and recurrent types in the future. In addition, analysis of mRNA in peripheral blood has been reported for biomarker discovery (49), therefore it is necessary to analyze expression profile of antisense transcripts in peripheral blood to find useful biomarkers.

Antisense transcripts have been demonstrated to be involved in gene regulation, but the function and mechanisms of antisense transcripts are still unclear. Functional analyses are required to define the mechanisms of tumorigenesis.

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