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ORIGINAL ARTICLE – CANCER RESEARCH

Identification of genes and pathways, including the CXCL2 axis, altered by DNA methylation in hepatocellular carcinoma

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Abstract

Purpose Recent genetic studies have suggested that tumor suppressor genes are often silenced during carcinogenesis via epigenetic modification caused by methylation of promoter CpG islands. Here, we characterized genes inactivated by DNA methylation in human hepatocellular carcinoma (HCC) to identify the genes and pathways involved in DNA methylation in hepatocellular carcinoma.

Methods Eight HCC-derived cell lines were treated with a DNA demethylating agent, 5-aza-2′-deoxycytidine. Additionally, 100 pairs of primary HCC and adjacent non-cancerous tissues as well as 15 normal liver tissues were analyzed by comprehensive gene expression analysis using microarrays. Moreover, gene set enrichment analysis identified the major molecular pathways associated with DNA methylation. Validation of gene expression and DNA methylation status was performed by real-time PCR after bisulfite modification.

Results We showed that CXCL2, an immune-related chemokine, expression was significantly downregulated in tumor tissues and also significantly upregulated by DAC treatment in cell lines. Furthermore, we observed a statistically significant difference in methylation status between normal liver tissues and tumor tissues ($P < 0.05$). In addition, tumors with higher CXCL2 expression included significantly more numbers of multiple tumors than the lower expression group.

Conclusions We identified CXCL2, an immune-related chemokine, decreased in hepatocellular carcinoma and the regulation mechanism may be controlled by methylation. Further studies should be warranted to examine if and to what extent the gene is actually suppressed by methylation and if there is a possibility that the CXCL2 axis plays a role for diagnosis and treatment of hepatocellular carcinoma.

Keywords Hepatocellular carcinoma · DNA methylation · Tumor suppressor genes · CXCL2 · Inflammation

Abbreviations

ATF3	Activating transcription factor 3
CGIs	CpG islands
CXCL2	Chemokine (C-X-C motif) ligand 2
DAC	5-aza-2′-deoxycytidine

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EMT	Epithelial–mesenchymal transition
FC	Fold change
FDR	False discovery rate
GRO	Growth-related oncogene
GSEA	Gene set enrichment analysis
HCC	Hepatocellular carcinoma
JUNB	Jun B proto-oncogene
KEGG	Kyoto Encyclopedia of Genes and Genomes
MSP	Methylation specific PCR
OS	Overall survival
PCR	Polymerase chain reaction
PTGS2	Prostaglandin-endoperoxide synthase 2
RFS	Recurrence-free survival
RMA	Robust multiarray average
SD	Standard deviation
TFBS	Transcription factor binding site
TGF-β	Transforming growth factor-β
THBS1	Thrombospondin 1
TSGs	Tumor suppressor genes

Introduction

After the completion of the Human Genome Project in 2003, transcriptomic, proteomic, epigenomic and bioinformatics analyses have been extensively utilized in the post-genome era. Epigenetics and cancer have been commonly studied with regard to activation of oncogenes, silencing of tumor suppressor genes, and multiple changes in cancer cells. The discovery of reversible epigenetic modifications has led to the identification a promising target of epigenetic therapy. An increasing number of studies have shown that not only genetic variations but also epigenetic modifications contribute to various diseases. The first identified and most commonly studied epigenetic marker is DNA methylation, which leads to abnormal silencing of genes and is crucial in the development of nearly all types of cancer (Laird and Jaenisch 1996; Jones and Takai 2001; Esteller 2008). Previous genetic studies suggested that tumor suppressor genes (TSGs) are often silenced during carcinogenesis via DNA methylation of promoter CpG islands (CGIs). Hypermethylation of promoter CGIs is an important mechanism for inactivation of tumor suppressor genes or tumor-related genes in cancer.

Many epigenetic drugs that can effectively reverse DNA methylation in cancer have been recently identified. Multiple experimental studies using cell culture analyses have shown that demethylation agents can reverse the silencing of genes due to methylation (Plumb et al. 2000). For example, it is possible to rescue the activity of methylated genes in cancer cell lines using 5-aza-2′-deoxycytidine (DAC) demethylating agents (Reuveni and Rosenthal 1979; Esteller 2008). DAC is incorporated into DNA and interferes with the activity of DNA methyltransferase, leading to genomic hypomethylation, and can reactivate silenced TSGs (Li et al. 1970). Further elucidation of the development of specific epigenetic modifications in cancer cells may assist in successfully altering the abnormal epigenetic changes.

Hepatocellular carcinoma (HCC) is the most common form of primary hepatic tumors and is one of the most common aggressive malignancies in humans. Rising incidence and mortality rates have been observed in many countries, especially some areas of Asia (Llovet et al. 2003). Because DNA methylation has recently been identified in several regulatory genes of HCC, the role of epigenetic deregulation in HCC is being increasingly recognized (Nishida et al. 2008; Huang et al. 2012; Ozen et al. 2013). Thus, a detailed investigation and characterization of genes inactivated by DNA methylation in HCC is necessary for the development of novel treatment options for HCC.

Over the past 10 years, inflammation has been associated with epigenetic reprogramming and has been shown to be one of the inducers of aberrant DNA methylation in specific types of cancer. Inflammation affects every aspect of tumor development and progression, as well as immune surveillance and responses to therapy. Thus, chemokines have been proposed as novel molecular targets for cancer, and many reports have demonstrated differences in chemokine receptors and functions between tumor cells and normal cells. High expression levels of several inflammation-related genes have been identified in prostate, gastric and colon cancer (Schwarze et al. 2005; Friederichs et al. 2005; Niwa et al. 2010; Doll et al. 2010). However, several studies showed low or absent expression of inflammation-related genes in breast cancer (Hromas et al. 1999; Basolo et al. 1993; Cao et al. 2013). The role of chemokines in tumor biology is complex; some chemokines act as angiogenic and tumor-promoting proteins, whereas others are angiostatic and inhibit tumor development.

In the present study, we investigated the major molecular pathways associated with DNA methylation in HCC and examined biological functions that may be strongly altered by DNA methylation. Our candidate genes may be possible target molecules for the development of anticancer therapeutics and may lead to improved diagnostic markers and treatment.

Materials and methods

Cell lines and DAC treatment

Eight established human HCC cell lines were used in this study. Huh1, Huh7, JHH2, JHH4, HepG2, HLE and HLF cell were obtained from the Human Science Research Resources Bank (Osaka, Japan). Hep3B cells were obtained from the

American Type Culture Collection (Manassas, VA, USA). The cell culture conditions have been previously reported (Tanaka et al. 2008; Yasen et al. 2009). All cell lines were maintained in Dulbecco's minimum essential medium (DMEM; Sigma, St. Louis, MO, USA) containing nonessential amino acids (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA) and were grown at 37 °C in 5% CO_2. After 48 h, cell lines were treated with 5 μM of DAC (Sigma) for the following 3 days. The medium and the drug were replaced every 24 h. As a control, cells were cultured in normal medium that was also replaced every 24 h.

Patients and specimens

A total of 100 pairs of HCC tissues and adjacent non-cancerous tissues were collected from 100 patients with HCC immediately after surgical resection. The adjacent non-cancerous tissues may be affected by cirrhosis or hepatitis. Additionally, 15 normal liver tissues were obtained from another set of 15 patients with metastatic liver cancer. All patients underwent surgical resection in the Department of Hepato-Biliary-Pancreatic Surgery between April 2003 and March 2008 at Tokyo Medical and Dental University Hospital. All tissue specimens were immediately snap-frozen in liquid nitrogen and stored at − 80 °C for further analysis. Histological diagnosis was made when two pathologists specializing in liver disease reached the same conclusion. None of the patients had received chemotherapy or radiation therapy before surgery. The patient characteristics and clinicopathological features are listed in Supplementary Table S1. The institutional review board approved the research project, and informed consent was obtained from all patients.

RNA extraction

Total RNA was extracted from HCC cell lines, cancer tissues, adjacent non-cancerous tissues and normal liver tissues using a RNeasy Mini kit (Qiagen, Valencia, CA, USA) and then treated with RNase-free DNase I according to the manufacturer's instructions. The integrity of RNA was assessed using the Agilent Bioanalyzer RNA Assay (Agilent Technologies, Palo Alto, CA, USA). All samples had an RNA integrity number greater than 5.0.

Gene expression analysis by DNA microarray

Using two micrograms of total RNA, cRNA was prepared using one-cycle target labeling and a control reagent kit (Affymetrix, Santa Clara, CA, USA). Hybridization and signal detection of HG-U133 Plus 2.0 arrays (Affymetrix) were performed according to the manufacturer's instructions. The gene expression profiles were normalized by the robust multiarray average (RMA) method with R statistical software version 2.12.1 together with the BioConductor package. We conducted separate normalization procedures for the microarray datasets obtained from the cell lines and the clinical specimens.

Welch's t-test was used to determine the statistical significance of differences in mRNA levels between the tumor and normal liver tissues. The data are displayed as the mean ± standard deviation (SD). The fold change (FC) values were calculated using the ratio of geometric means of the gene expression levels between normal cell lines and cell lines treated by DAC and the geometric means of gene expression levels between tumor and normal liver tissues.

To identify genes associated with the expression of CXCL2 in HCC tissues, the Wilcoxon rank-sum test was performed to estimate the significance of gene expression differences between the CXCL2 (+) and CXCL2 (−) groups for each of the 54,613 probes on the Human Genome U133 Plus 2.0 array. Obtained *P* values from the multiple hypothetical testing were adjusted by the false discovery rate (FDR) (Benjamini and Hochberg 1995). Genes with $FDR < 0.25$ were considered for further analysis. The FC values were calculated using the ratio of the geometric means of the gene expression levels between these two groups.

Gene set enrichment analyses

For investigation of the biological background associated with gene expression changes between DAC-treated and untreated cells, gene set enrichment analysis (GSEA) version 2.0.7 with MSigDB gene sets version 3.0 was used. Gene set category C2 Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto 2000), which is based on the KEGG PATHWAY database was used (http://www.genome.jp/kegg/pathway.html). Gene sets satisfying both $P < 0.05$ and FDR < 0.25 were considered statistically significant. We selected common gene sets that were upregulated in at least four cell lines.

TaqMan MGB probe real-time PCR

Two micrograms of RNA from eight cell lines and frozen tissues were reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. TaqMan gene expression assays for chemokine (C-X-C motif) ligand 2 (CXCL2) (Hs_00601975_m1) were performed using a TaqMan Fast Universal PCR master mix on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). RT-PCR reactions (20 μL) contained 10 μL of 2 × AB TaqMan Fast Universal PCR Master Mix, 1 μL of the relevant 20x assay, 2 μL of target cDNA and 7 μL of dH_2O and were performed under the following conditions:

one cycle of 20 s at 95 °C, and 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Reactions were performed in triplicate. For quantitative analysis of specific mRNA expression, cycle threshold values were analyzed using 7500 SDS software. For each sample, expression of the CXCL2 gene was normalized to the expression of the 18S rRNA control gene, and fold differences between cancer and non-cancerous tissues were calculated using the $\Delta\Delta C_T$ method (Schmittgen and Livak 2008). (https://tools.thermofisher.com/content.sfs.manuals/cms_053412.pdf).

Quantitative real-time RT-PCR

Two micrograms of RNA from cells and tissues was reverse-transcribed to cDNA with the High-Capacity cDNA Reverse Transcription System (Applied Biosystems). Quantitative PCR was performed using the SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences are shown in Supplementary Table S2. The PCR products were separated by 2% agarose gel electrophoresis, and the DNA bands were visualized under ultraviolet light for photographing.

Sodium bisulfite modification and PCR

We obtained genomic DNA from cancer cell lines as well as cancerous, adjacent non-cancerous and normal tissues using a QIAamp Mini Kit (Qiagen) following the manufacturer's instructions. To determine the methylation pattern of 5′ CGIs of the CXCL2 gene promoter in HCC cell lines and cancer tissues, we modified 10 μg of DNA using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Modified DNA was amplified immediately because of the unstable situation of CT conversion. Bisulfite-treated DNA was stored at − 20 °C until use. We searched CGIs in the CXCL2 promoter region based on the NCBI Reference Sequence (GenBank accession ID: NM_002089) using CGI searcher (http://www.cpgislands.com) with the default settings (%GC > 55%, Observed CpG/Expected CpG > 0.65). CGIs in the CXCL2 promoter region searched on promoter structure of CXCL2 shown in Supplementary Fig. S1. The primers were designed by Methyl Primer Express v1.0 software (Applied Biosystems). The primer sequences are shown in Supplementary Table S2. The FastStart High Fidelity PCR system (Roche Applied Science, Germany) was used for amplifying CGIs in the promoter region with the expected product of 560 bp. The PCR conditions for primers M13F1-BSF1 and M13R1-BSR1 were as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 amplification cycles of 95 °C for 15 s, 1 min at 55 °C and 30 s at 72 °C, and a final extension at 72 °C for 3 min. The final PCR products were digested and electrophoresed on 2 % agarose gels, and DNA bands were visualized under ultraviolet light for photographing.

Methylation-specific PCR analysis

After bisulfite conversion, methylated and unmethylated DNA sequencing was performed using two sets of specific primers spanning a region 125 bp upstream of the first exon of CXCL2 (chr4: 74,097,035–74,099,293). The primer pairs are shown in Supplementary Table S2. The first methylation-specific PCR using specific primer sets was performed with sensitive and reliable high-fidelity hot-start PCR (HotStar HiFidelity PCR Kit, QIAGEN) under PCR conditions as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 95 °C for 15 s, 1 min at 58 °C and 30 s at 72 °C, and a 3 min final extension at 72 °C.

The other non-methylation PCR using specific primer sets was performed with a FastStart HiFi PCR System containing 2.5 U of FastStart High Fidelity Enzyme Blend in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) under PCR conditions as follows: 2 min at 95 °C, 40 cycles of 30 s at 95 °C, 50 s at 50 °C and 1 min at 72 °C, and final 72 °C for 3 min. Methylation status of the CXCL2 gene was analyzed only in 51 paired cases of primary HCC tissues and adjacent non-cancerous tissues. The PCR products were electrophoresed on 2 % agarose gels, and DNA bands were visualized under ultraviolet light for photographing.

Statistical analysis

The categorical data and relationships between CXCL2 expression and clinicopathological variables were assessed by Welch's *t* test. The data are displayed as the mean ± SD. Differences in mRNA levels between the groups and the association between clinicopathological factors were analyzed using paired Welch's *t* tests. Survival was analyzed using the log-rank test. A value of *P* is less than 0.05 was considered statistically significant. All statistical analyses were performed using R statistical software (version 2.12.1).

Results

Genes and molecular pathways altered after demethylation of HCC cell lines

To identify candidate genes that were silenced by DNA methylation in HCC, we examined eight HCC cell lines treated with the demethylating agent DAC compared with the control cells, using microarrays. Among 54,613 probe sets included in the HG-U133 Plus 2.0 array, we identified a total of 27 genes that were significantly upregulated more

than threefold in at least six cell lines by DAC treatment (Supplementary Table S3). Among them, cytokines involved in immune responses, including interleukin 8 (IL8), were upregulated. Similarly, other interleukin proteins, including interleukin 32 (IL32), and CXC chemokines, such as CXCL1, 2, 3 and 5, were also upregulated. We also confirmed the 27 genes expression pattern in the clinical samples (data not shown). The gene yippee-like 2 (YPEL2), Chromosome 8 open reading frame 4 (C8orf4) and CXCL2 expression were downregulated in the tumor tissues compared with the normal liver tissues. Notably, the common gene that was downregulated in the cell lines and tumor tissues, and upregulated by the DAC treatment, was CXCL2.

Moreover, ontological analysis using GSEA and the KEGG PATHWAY database identified the molecular interactions and reactions associated with HCC-derived cell lines after the DAC treatment. Pathways associated with the immune responses, which included "cytokine–cytokine receptor interaction", "Nod-like receptor signaling pathway", "chemokine signaling pathway" and "p53 pathway" were commonly upregulated by the DAC treatment (Supplementary Table S4). The results suggest that these genes and pathways may play important roles through inactivation by the DNA methylation.

Selection of candidate genes using clinical samples

To further select candidate genes downregulated by the DNA methylation in HCC, we applied a combination of criteria for selection of genes by comparing the gene expression profiles in the clinical samples (Supplementary Fig. S2). First, using cell line samples, 445 probe sets satisfied twofold upregulation by the DAC treatment in at least four out of eight cell lines. Moreover, the ontological analysis using the KEGG PATHWAY database indicated that reactions associated with immune responses were commonly upregulated. In a representative pathway of a cytokine–cytokine receptor pathways (accession ID: hsa04060), common upregulation of immune response genes was observed in DAC-treated cells (data not shown). Additionally, 1248 probe sets satisfied the criteria of P value less than 0.01 and more than twofold downregulation in tumor tissues ($n = 100$) compared with normal liver tissues ($n = 15$). In total, there were 44 probe sets that met both criteria. The gene expression patterns of the candidate probe sets are shown in Fig. 1.

As described above, GSEA indicated that downregulation of immune response pathways was induced by DNA methylation in DAC-treated HCC cells. Moreover, including the 876 genes associated with the 11 upregulated pathways, there were only three genes considered putative hypermethylated genes in HCC, as shown in Fig. S2. These genes included CXCL2, prostaglandin-endoperoxide synthase 2 (PTGS2) and thrombospondin 1 (THBS1).

Among them, CXCL2 is involved in various biological progresses, such as angiogenesis, inflammation and cancer biological behaviors. It is associated with immune response and is an important factor in inhibition of carcinogenesis (Doll et al. 2010). Therefore, downregulation of CXCL2 may promote immune escape of cancer cells (Hromas et al. 1999; Basolo et al. 1993; Cao et al. 2013). Moreover, previous studies examined the role of CXCL2 in HCC and the regulation of its expression by miRNAs. CXCL2 enhanced liver cancer cell proliferation, migration and invasion (Zhu et al. 2014; Song et al. 2015). Thus, we hypothesized that CXCL2 could be one of the key molecules downregulated in cancer progression by DNA methylation and focused on the CXCL2 gene for subsequent analyses.

Induction of CXCL2 gene expression by DAC treatment

To confirm the role of DNA methylation in the regulation of CXCL2 expression, we examined eight HCC cell lines (HepG2, Hep3B, Huh1, Huh7, JHH2, JHH4, HLE, and HLF) using qRT-PCR. The results showed that CXCL2 expression was significantly upregulated by DAC treatment in all eight cell lines (Fig. 2). Moreover, the expression levels in the cell lines HepG2 and HLF increased more than twofold compared to those of untreated cells. These results were consistent with the microarray data which suggested that potential silencing of CXCL2 expression may occur through hypermethylation of the CXCL2 gene promoter in HCC cells.

CXCL2 mRNA expression levels are downregulated in tumor tissues compared to those of adjacent non-cancerous tissues and normal liver tissues

To validate the microarray results, we measured the mRNA levels of CXCL2 using real-time RT-PCR in normal liver tissues and 81 pairs of tumor and adjacent non-cancerous tissues (randomly selected from the above 100 pairs). Agarose gel electrophoresis of PCR products confirmed the amplification of CXCL2 in the normal tissues. Figure 3a showed typical two samples of normal tissues, termed C14MN and C20MN, and tumor tissues L42T and L47T. Moreover, we confirmed downregulation tendency of the expression levels of CXCL2 mRNA in normal tissues, adjacent non-tumor tissues and tumor tissues, and the expression in normal tissues were significantly higher than tumor tissues ($P < 0.001$; Fig. 3b). These results were consistent with the DNA microarray data showing downregulation of CXCL2 in tumor tissues.

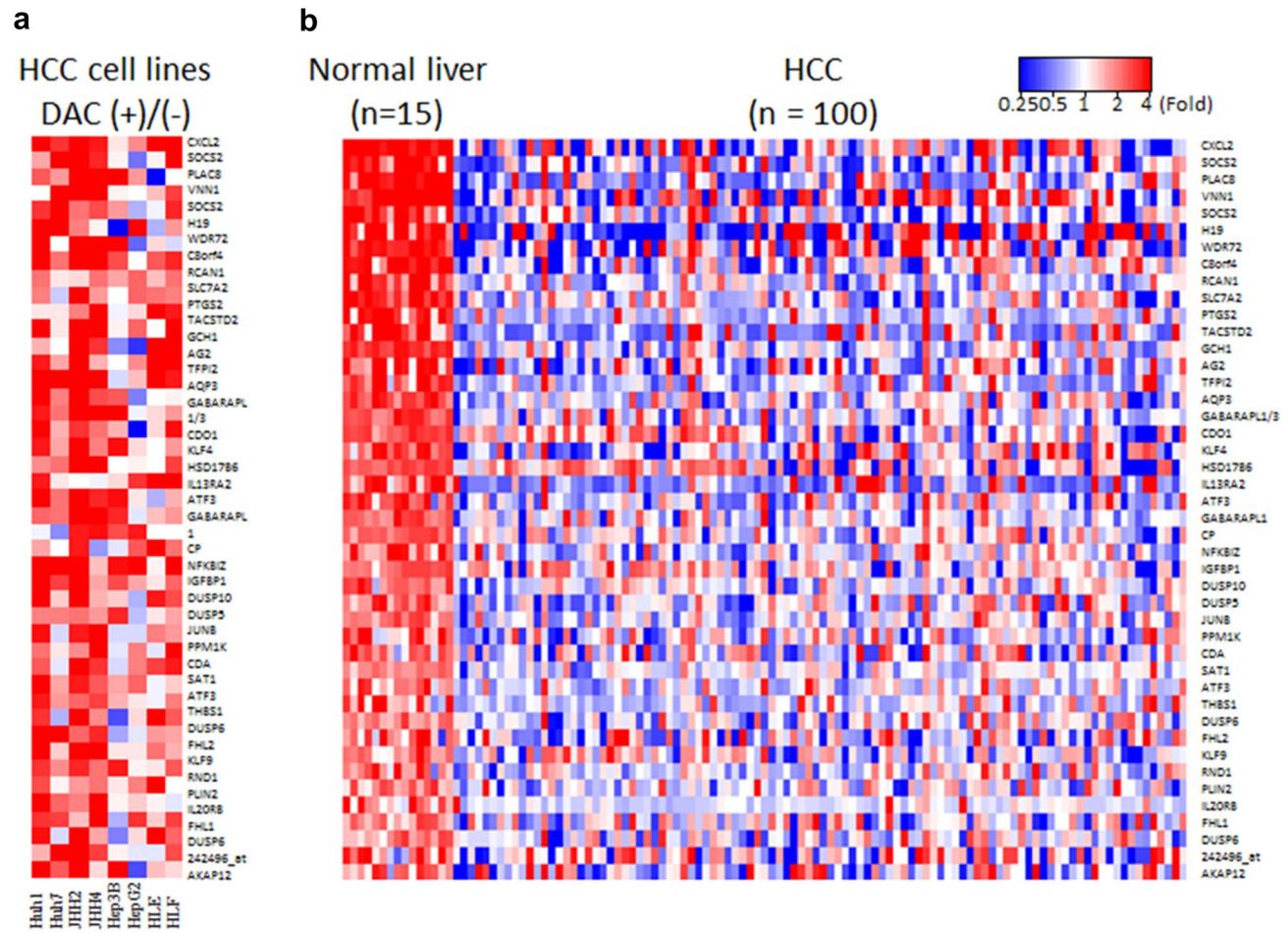

Fig. 1 Gene expression patterns of 44 candidate probe sets. **a** Gene expression changes after DAC treatment. The ratios between DAC-treated and control cells are shown. **b** Gene expression patterns in the 15 normal and the 100 hepatocellular carcinoma tissues. For each gene, the ratio of the mean expression level is shown as the gradient color from blue to red

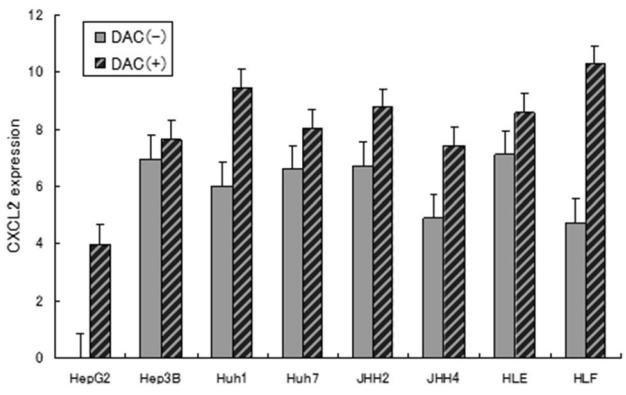

Fig. 2 The expression of CXCL2 mRNA in hepatocellular carcinoma cell lines and the effect of DAC treatment are shown. In all the eight cell lines, CXCL2 expression was upregulated by DAC treatment, fully for HepG2, approximately twofold for HLF and to some extent for the other six cell lines

Next, the correlation between gene expression levels of CXCL2 by a TaqMan gene expression assay and the microarray was examined using the 81 cases tumor tissues. There were significant positive correlations between the DNA microarray and TaqMan gene expression assay results for CXCL2 ($R = 0.488$, $P < 0.001$), as shown in Supplementary Fig. S3.

Methylation analyses of the CXCL2 promoter in HCC and patient survival

Aberrant promoter methylation of the CXCL2 gene was determined by sodium bisulfite modification and methylation-specific PCR (MSP) (Herman et al. 1996). MSP was performed in eight cell lines, 15 normal liver tissues and 51 pairs of tumor and adjacent non-cancerous tissues (randomly selected from the above 100 pairs) after a detailed methylation analysis by bisulfite sequencing PCR. Figure 4a, b showed the specific DNA methylation

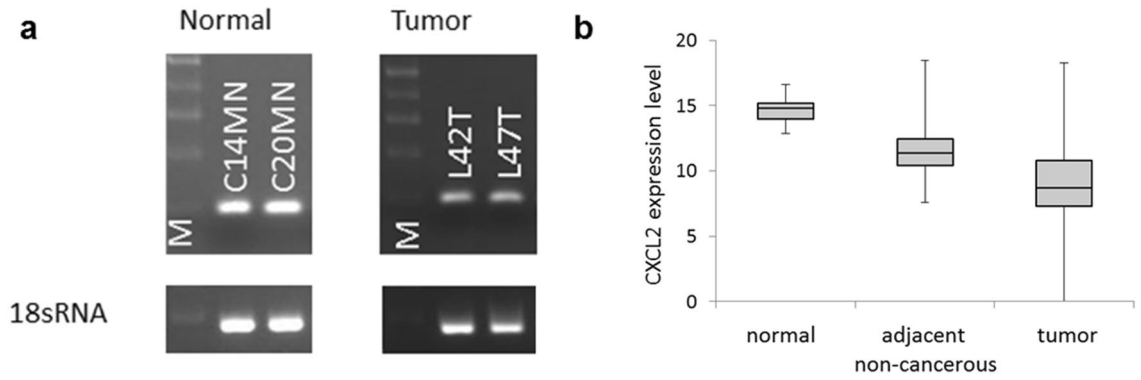

Fig. 3 Validation of CXCL2 mRNA levels in normal tissues and in tumor tissues by real-time RT-PCR (**a**; typical four samples are shown). The CXCL2 mRNA levels were significantly upregulated in normal tissues ($n=15$), compared with 81 pairs of tumor and adjacent non-tumor tissues (**b**)

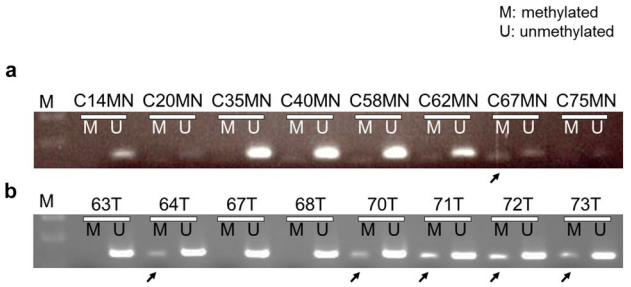

Fig. 4 Methylation-specific PCR (MSP) analysis of the CXCL2 promoter in normal tissues and tumor tissues. Representative data of the CXCL2 promoter sequence after bisulfite modification. Specific DNA methylation of CGIs was detected in 6.7% (**a**) of normal liver tissues and 68.6% (**b**) in HCC tissues. Arrows showed positive methylation bands, though some were weak, and all the other methylation bands were negative

Table 1 Correlation between normal tissues with HCC in methylation status

Methylation status	Normal tissues $n=15$	HCC $n=51$	P value
Variables			
Absent	14	16	<0.05
Present	1	35	

The significance of the difference between groups in the table was assessed by Fisher's exact test

of CGIs was detected in 1 out of 15 (6.7%) normal liver tissues (MN) and in 35 out of 51 (68.6%) HCC tissues (*T*), respectively. There was a statistically significant difference in methylation status between normal tissues and tumor tissues ($P < 0.05$ by Fisher's exact test; Table 1). The results of DNA methylation analysis in tumor tissues with higher expression of CXCL2 were consistent with the previous DNA microarray results. We also compared the overall survival (OS) and the recurrence-free survival (RFS) in the 51 cases between the higher and the lower methylation groups (Supplementary Fig. S4). There were no significant differences in both overall and recurrence-free survival (OS, $P = 0.987$; RFS, $P = 0.391$).

Statistical analysis of clinicopathological correlations

To clarify the clinical roles of CXCL2, we analyzed the correlations between the expression level of CXCL2 and the clinicopathological parameters of HCC patients. The 81 tissue samples were divided into two groups based on the CXCL2 expression level. The high-expression group included 41 samples from patients whose tumor tissues expressed CXCL2 mRNA above the median level. Similarly, the low-expression group included 40 patients whose tumor tissues expressed CXCL2 mRNA below the median value. No statistically significant differences were observed in age, sex, size and stage of the tumor or other factors between the two groups. However, there was a statistically significant difference in CXCL2 expression levels in tumor multiplicity ($P = 0.048$; Table 2).

Discussion

Previous gene expression studies using DNA microarrays have shown that chemokines of the CXC family were specifically upregulated in colorectal cancer (Doll et al. 2010; McLean et al. 2011). GRO-2 (CXCL2) and GRO-3 (CXCL3) belong to the growth-related oncogene (GRO) subgroup of chemokines that was reported to be enhanced in premalignant adenomas. In previous reports, GRO-3 was strongly overexpressed in liver metastases compared with primary tumors (Doll et al. 2010). However, GRO-2

Table 2 Clinicopathological correlation of chemokine (C-X-C motif) ligand 2 (CXCL2) expression in human hepatocellular carcinoma (HCC)

Clinicopathologic factors	CXCL2 expression in HCC ($n=81$)			*P* value
	Total $n=81$	High $n=40$	Low $n=41$	
Age in years				0.432
< 50 years	6	4	2	
≧ 50 years	75	36	39	
Gender				0.141
Male	67	36	31	
Female	14	4	10	
Background of liver				1.000
Normal liver	1	0	1	
Chronic hepatitis	39	20	19	
Liver cirrhosis	41	20	21	
Viral infection				1.000
HBV	18	9	9	
HCV	39	19	20	
NBNC	24	12	12	
PVIKAII				0.375
< 100 mAU/mL	44	24	20	
≧ 100 mAU/mL	37	16	21	
Preoperative AFP level				0.404
< 400 ng/mL	66	31	35	
≧ 400 ng/mL	15	9	6	
Histopathologic grading				0.588
Well	24	13	11	
Moderately	41	21	20	
Poorly	16	6	10	
Tumor size				0.485
< 5 cm	53	28	25	
≧ 5 cm	28	12	16	
Tumor number				0.048*
Solitary	59	25	34	
Multiple	22	15	7	
Tumor stages				1.000
I + II	39	19	20	
III + IV	42	21	21	

AFP alpha-fetoprotein

*$p < 0.05$. The significance of the difference between groups in the table was assessed by the Fisher's exact test

expression was downregulated in breast cancer (Gupta et al. 2007). Furthermore, many chemokines, including CXCL1, CXCL2, CXCL6 and CXCL8, were highly expressed in normal breast tissues and were lost in tumor tissues (Porter et al. 2001). This strongly suggested that inflammatory reaction at the breast tumor site enhances tumor growth and progression. In our study, CXCL2 expression was significantly upregulated by DAC treatment in HCC cell lines, and CXCL2 showed lower expression in tumor tissues compared with adjacent non-cancerous tissues and normal tissues.

CXCL2, also known as growth-regulated protein beta and macrophage inflammatory protein 2-alpha, is a small secreted cytokine that belongs to the CXC chemokine family. CXCL2 mediates chemotaxis by interacting with a cell surface chemokine receptor called CXCR2. CXCR2 regulates immune functions predominantly through chemoattraction of neutrophils and is expressed at sites of inflammation (Ha et al. 2010). Furthermore, CXCL2 is also involved in the initiation and promotion of carcinogenesis.

A previous study showed that inflammation is involved in the promotion and progression of tumors. Therefore, chemokines have been proposed as novel molecular targets.

CXCL2 was the first major chemokine produced by endotoxin-treated macrophages, and the principal role of CXCL2 is chemotaxis of neutrophils (Blengio et al. 2013). CXCL2 has also been shown to be induced by receptor activator of NF-κB ligand through the NF-κB signaling pathways in osteoclasts (Ha et al. 2010). According to the promoter prediction results in GSEA software (Subramanian et al. 2005) that are based on the TRANSFAC database, NF-κB, FREAC7, HFH8, FOXJ2 and POU3F2 were predicted to have transcription factor binding site (TFBS) in the promoter region of CXCL2. Although expression levels of these five transcription factors were all downregulated by DAC treatment, CXCL2 expression was significantly upregulated by DAC. Hence, these transcription factors may not directly affect the expression of CXCL2 in HCC cell lines. Other transcription factors that were not predicted by TRANSFAC may regulate CXCL2 expression, but further investigation is necessary to clarify the exact regulatory mechanism of CXCL2. Taken together, the data indicated that upregulation of CXCL2 is possibly caused by DNA demethylation.

CXCL2 expression was also significantly upregulated by DAC treatment in HCC. We found that the expression levels of CXCL2 were downregulated in HCC compared with those of normal liver tissues and adjacent non-cancerous tissues (Fig. 3). To determine whether the expression of CXCL2 is regulated by methylation in the CpG islands, the methylation status of the CXCL2 promoter was analyzed by bisulfite modification; however, the results were ambiguous. Because the candidate gene CXCL2 might be an important molecule for immune escape in HCC, further bisulfite sequencing analyses using additional samples should be performed to clarify the methylation patterns in HCC patients.

The study also identified two other candidate genes commonly methylated in HCC. PTGS2, also known as COX2, is not expressed under normal conditions in most cells, but is expressed during inflammation when prostaglandins are upregulated. The expression of PTGS2 is upregulated in many cancers, and CGIs in PTGS2 were hypermethylated in prostate cancer (Yegnasubramanian et al. 2004). THBS1 has been shown to play roles in platelet aggregation, angiogenesis and tumorigenesis. More recent data demonstrated that THBS was completely unmethylated in normal liver tissues and hypermethylated in cancer cell lines (Li et al. 1999). Several studies have indicated that THBS1 may have tumor-suppressive properties, probably through inhibition of neovascularization. Further studies should be performed on these two candidate genes.

In addition, several remaining genes were associated with transcriptional regulation. For example, the transcription factor Jun B Proto-Oncogene (JUNB) is reported to play an important role in transforming growth factor-β (TGF-β)-induced epithelial–mesenchymal transition (EMT) and profibrotic responses. The role of JUNB in the tumor suppressor function of TGF-β contradicts the current idea of EMT as a process that underlies tumor invasion and metastasis (Gervasi et al. 2012). Activating transcription factor 3 (ATF3) is also a highly versatile stress sensor for inflammatory reactions (Gilchrist et al. 2006) and has been reported to be overexpressed in various tumors, including those of the prostate and breast (Pelzer et al. 2006).

There are some limitations in our study. First, although the CXCL2 is certainly the most important gene in this study, other related genes such as PTGS2 and THBS1 had better be analyzed. Second, the methylation status of the CXCL2 promoter was examined but the results were not definite. Further investigations using more number of samples/cell lines may be needed. Third, we employed only specimens of Japanese patients. Since the etiology of HCC is different in different countries, investigation using samples from different ethnicities may provide other results.

Conclusion

In conclusion, we verified that CXCL2 expression was significantly downregulated in tumor tissues and also significantly upregulated by DAC treatment in cell lines. Furthermore, we observed a statistically significant difference in methylation status between normal liver tissues and tumor tissues of the liver. CXCL2 may be a potential target molecule for development of anticancer strategies and improved diagnostic markers and treatment. The genes and pathways identified in this study may also play important roles in carcinogenesis and disease progression of HCC through inactivation by DNA methylation.

Authors' contributions All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization: SS, MY and HT Methodology, KM, MY and TK Investigation, SS, KM and MY Formal Analysis, SS and KM Resources, KM, TK and HT Writing—Original Draft, SS Writing-Review & Editing, KM and YI Visualization, SS Supervision, KM and YI Funding Acquisition KM and YI.

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Compliance with ethical standards

Conflict of interest Yuichi Ishikawa received research grants from Daiichi Sankyo Co., Ltd. and Ono Pharmaceutical Co., Ltd., as well as he is a consultant of Fujirebio Inc. All other authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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