Estrogen receptor 1 gene as a tumor suppressor gene in hepatocellular carcinoma detected by triple-combination array analysis

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Abstract. Hepatocellular carcinoma (HCC) is one of the top five causes of cancer-related deaths worldwide. Recent developments in the treatment of HCC remain insufficient to cure unresectable disease or to prevent HCC. Consistent efforts are, therefore, needed to deepen understanding of pathogenesis of the disease. Genome-wide gene expression profile analyses can now detect various candidate genes that are modified by HCC. We have developed a new technique to identify tumor suppressor genes, triple-combination array analysis, which combines gene expression profiles, single nucleotide polymorphism and methylation arrays to identify genes with altered expression. Using HCC tissue samples, triple-combination array analysis was performed to identify a candidate tumor suppressor gene. Subsequently, samples from 48 HCC patients were subjected to quantitative polymerase chain reaction (qPCR) and methylation-specific PCR to further elucidate clinical relevance of the gene. Estrogen receptor 1 (ESR1) was detected as a candidate tumor suppressor gene. Of the 48 clinical samples, 40 (83.3%) showed ESR1 promoter hypermethylation. In 24 (50%) HCC samples, the expression levels of the ESR1 gene was decreased by >90%. The decreased expression was significantly related to high liver damage score, pathological invasion of the intrahepatic portal vein, the size of tumor (>3 cm in diameter) and hepatitis B virus infection. The present study represents another example that triple-combination array is a convenient technique for detecting genes with altered expression in disease. The ESR1 gene was identified as a candidate tumor suppressor gene in HCC and further validation is warranted.

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Introduction

Hepatocellular carcinoma (HCC) is one of the top five causes of cancer-related deaths worldwide (1). Hepatitis B and C viruses are major risk factors for HCC and many studies of HCC associated with viruses have been undertaken. Recent developments in imaging have enabled the detection of early-stage HCC and multidisciplinary treatment of HCC has greatly improved the survival rate; however, recurrence of HCC remains prominent. One reason for this high rate of recurrence is that cancer is a genetic disease of somatic cells arising from an accumulation of genetic mutations. Therefore, in order to improve the prognosis, it is important to find the genetic markers occurring in recurrent or metastatic HCC.

The development of high-throughput technologies such as gene expression microarrays and single nucleotide polymorphism (SNP) arrays that can simultaneously screen thousands of genes has enabled wide and comprehensive identification of alterations in gene expression caused by oncogenesis (2-4). These technologies have revealed an enhanced characterization of individual tumors concerning metastatic potential, compared with that provided by traditional clinicopathological methods.

We have previously reported a novel method named double-combination array analysis that combines SNP arrays with gene expression arrays (5-10). In addition, we have hypothesized that the decrease in gene expression is due to hypermethylation of the CpG islands. Aberrant DNA methylation of the promoter and other genomic regions can lead to changes in gene expression. However, our double array analysis does not focus on the presence of hypermethylation. Therefore, we additionally performed methylation array analysis by using the IlluminaInfiniumHumanMethylation 27 BeadChip platform (Illumina, San Diego, CA, USA) to comprehensively evaluate the methylation status. We used data from all three analyses to detect the aberrant gene

expression in tumor tissue. We called this approach triple-combination array analysis.

In the assessment of the data from the triple-combination array analysis, estrogen receptor 1 (ESR1) gene was identified as one of promising candidates for a novel tumor suppressor gene. Estrogens play important roles, including oncogenic role in various organs of which breast cancer is a well-known example. Moreover, estrogens have antifibrotic effects and are protective factors for the progression of fibrosis in patients with chronic hepatitis. Thus, we selected ESR1 as a candidate tumor suppressor gene in the present study.

Materials and methods

Sample collection and DNA preparation. Nine HCC cell lines (HuH1, HuH2, HuH7, HepG2, Hep3B, HLE, HLF, SK-Hep1 and PLC/PRF/5) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in RPMI-1640, supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37˚C.

A 68-year-old woman with chronic hepatitis C was diagnosed as having HCC in the right lobe and underwent liver resection, and the tumor was pathologically diagnosed as HCC. The total RNA and DNA were extracted from her tumor and non-tumor tissues. Total RNA was sent to the manufacturer of Affymetrix to prepare it for expression array analysis, and genomic DNA was used for SNP array analysis, and bisulfite-converted DNA was used for the IlluminaInfiniumHumanMethylation 27 BeadChip methylation array analysis.

HCC tissues and corresponding normal tissues were obtained from 48 patients who had undergone liver resection at Nagoya University Hospital, Japan, between 1994 and 2001. Their ages ranged from 39 to 77 years (62.4±7.9 years: means ± standard deviation) and the male-to-female ratio was 43:5. Thirty-eight patients had hepatitis C and seven had hepatitis B. The median length of follow-up was 64.5 months (range 17.9-105.9 months). All tissues collected were diagnosed pathologically as HCC. Written informed consent, as required by the institutional review board, was obtained from all patients. The tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C until analysis. Genomic DNA was obtained from the tissue samples by digestion with proteinase K, followed by phenol/chloroform extraction.

RNA isolation, microarray and gene chip Affymetrix procedures. The expression array and SNP array analysis were performed, as previously described (5-10), using total RNA and DNA extracted from the 68-year-old woman's tissue samples.

Methylation array platform. Methylation array analysis was performed as described previously (12,13).

RT-PCR. The expression of ESR1 mRNA was analyzed by RT-PCR and real-time RT-PCR. After total RNA (10 μg) was isolated from nine HCC cell lines and primary HCC tissues and normal tissues were used to generate cDNA, they were amplified by PCR primers for ESR1 sense (S) (5'- CCGGCTCC GTAAATGCTACG-3' in exon 10) and antisense (AS) (5'-TCC AGCAGACCCCACTTCAC-3' in exon 11), which amplified a 133-bp product. RT-PCR amplification consisted of 36 cycles of 94˚C for 12 sec, 60˚C for 8 sec and 72˚C for 8 sec, after the initial denaturation step (94˚C for 5 min). GAPDH (TaqMan, GAPDH Control Reagents; Applied Biosystems) was used as a control reference. Each PCR product was loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under ultraviolet (UV) illumination.

Quantitative PCR. PCR reactions were performed by the SYBR Green PCR Core Reagents kit (Applied Biosystems) under the following conditions: 1 cycle at 95˚C for 10 min, then 40 cycles at 95˚C for 15 sec and at 60˚C for 30 sec. Real-time detection of the SYBR Green emission intensity was conducted with an ABI PRISM 7000 Sequence Detector (Applied Biosystems). The primers used for this PCR were the same primer pairs as used for the RT-PCR described above. For standardization, expression of GAPDH in each sample was quantified. Quantitative RT-PCR was performed at least three times, including no-template samples serving as a negative control. The expression of ESR1 was normalized by dividing the amount of ESR1 expression by GAPDH expression for each sample.

Methylation-specific PCR (MSP) and unmethylation-specific PCR (UNMSP). DNA from HCC cell lines, the primary tumor and corresponding normal specimens were subjected to bisulfite treatment. Briefly, 2 μg DNA was denatured by NaOH and modified by sodium bisulfite. Then, DNA samples were purified with the Wizard purification resin (Promega, Madison, WI, USA), treated again with NaOH, precipitated with ethanol and resuspended in water. The primer pairs for detecting methylation targeted the ESR1 promoter region near exon 3 were as follows: S (5'-TTCGTCGGGTCGTTCGGTTT-3') and AS (5'-ATATCCCGCCGACACGCGAA-3'), which amplified an 81-bp product. Primers for unmethylated detection targeted the same promoter region: S (5'-AGTTGGTGGAGGGTGT TTGT-3') and AS (5'-CACATATCCCACCAACACAC-3') and amplified a 122-bp product. Each PCR product was loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under UV illumination.

5-Aza-2'-deoxycytidine (5-aza-dC) treatment. To confirm that the promoter hypermethylation was responsible for the silencing of the gene expression, nine HCC cell lines were treated with a DNA methylation inhibitor, 5-aza-dC (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured for 6 days with the medium replaced on days 1, 3 and 5. After incubation, cells were collected, RNA was extracted and RT-PCR was performed as described above.

Sequence analysis. Genomic bisulfite-treated DNA of HCC cell lines was sequenced and PCRs were performed on each sample. The primer pair for the sequence was in the ESR1 promoter region for forward primer and in exon 3 for reverse primer: S (5'-TTTGGAGTGATGTTTAAGTT-3') and AS (5'-CCACCTAAAAAAAAAACACA-3'), which amplified a 288-bp product. The PCR amplification consisted of 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec and 60˚C for 4 min, following the initial denaturation step (96˚C for 1 min). PCR products were purified directly using the QIA Quick Gel extraction kit

Table I. Expression array analysis of ESR1 gene.

Gene symbol	Log_2 ratio	Normal signal	Detection	Tumor signal	Detection	Probe ID	Chromosomal location
ESR1	-2.5	295.8	P	55	P	HU133p2_14673	6q25.1

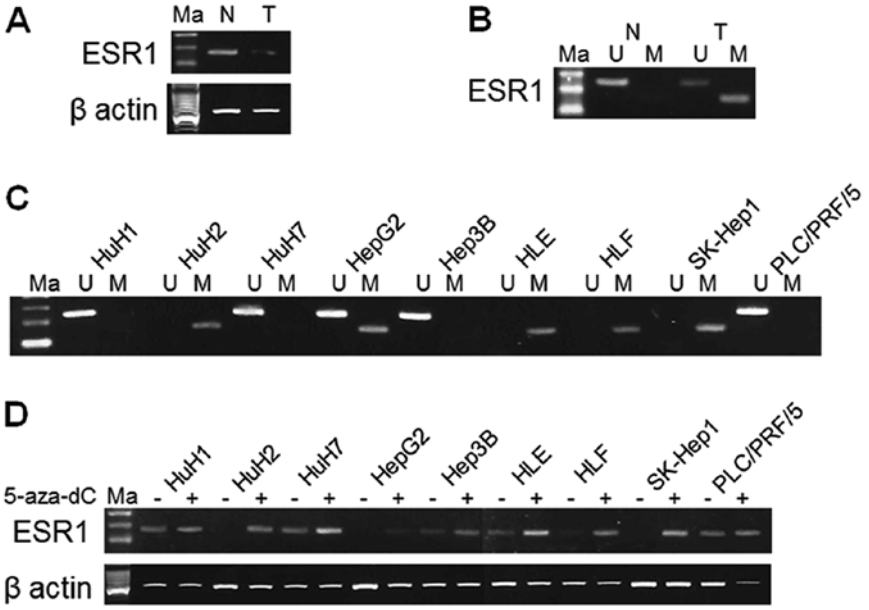

Figure 1. Results of RT-PCR, MSP and UNMSP in ESR1 gene, which produced a product of 133, 81 and 122 bp, respectively. (A) RT-PCR used cDNA obtained from the 68-year-old woman confirmed that ESR1 gene was downregulated. (B) Hypermethylation was confirmed by MSP in tumor tissue obtained from the 68-year-old woman donor. (C) Methylation status of ESR1 gene in HCC cell lines. HuH2, HepG2, HLE, HLF and SK-Hep1 cells showed hypermethylation. Unmethylation was shown in HuH1, HuH7, HepG2, Hep3B and PLC/PRF/5 cells. (D) Expression of ESR1 in HuH2, HepG2, HLF and SK-Hep1 cells displayed reactivation after 5-aza-dC treatment. ESR1, estrogen receptor 1; Ma, marker; N, normal tissue; T, tumor tissue; M, MSP; U, UNMSP.

(Qiagen, Hilden, Germany). The purified DNA fragments were subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA, USA). Six cloning samples were picked out from two HCC cell lines (HuH7 and SK-Hep1). Each cloning DNA was mixed with the specific primer (M13) and Cycle Sequence Mix (ABI PRISM Terminator v1. 1 Cycle Sequencing kit; Applied Biosystems, Foster City, CA, USA). We performed sequence analysis using an ABI PRISM 310 genetic analyzer (Applied Biosystems) and sequence electropherograms were generated by ABI Sequence Analysis software 5.1.

Statistical analysis. Continuous variables are expressed as medians (range) and comparisons were made using the Mann-Whitney U test. Categorical variables were compared using χ^2 or Fisher's exact tests, where appropriate. Overall and disease-free survival rates were analyzed by Kaplan-Meier and log-rank tests. All statistical analyses were performed using Jump 9 (SAS Institute Inc. Cary, NC, USA). The level of statistical significance was set at $P<0.05$.

Results

Expression, SNP and methylation arrays. To reveal potential tumor suppressor genes involved in HCC, expression array chips were used to identify genes for which expression was lower in HCC compared with normal tissue. Aberrant gene expression between HCC and normal tissue was ranked on the basis of change in intensity. We consequently identified that the ESR1 gene had greatly reduced expression in HCC tissues at the high level of -2.5 (log_2 ratio) (Table I). RT-PCR that used cDNA obtained from the 68-year-old woman confirmed ESR1 gene was downregulated (Fig. 1A). Focusing on the ESR1 gene, by means of the SNP-Chip array, we detected deletions in 3q, 8p, 11q, 12q, 16p, 17p and 19p, as well as in the X chromosomes and chromosomal gains in 1q, 3q, 11q, 12p and 12q. Interestingly, there were no abnormalities in the copy number of chromosome 6, where ESR1 is located (Fig. 2). SNP array data of the ESR1 locus were analyzed and displayed a high number of SNPs. Twenty-five SNPs showed a heterozygous AB allele in both normal and tumor tissues, indicating that both alleles were retained at the locus (Table II). These results suggest that ESR1 expression was reduced without loss of heterozygosity or deletion. In the methylation array analysis, the continuous β values were 0.775 for HCC tissue versus 0.093 for normal tissue, indicating high methylation in the former (Table III). Based on these findings, many CpG islands were detected when the sequence of the promoter region of ESR1 was examined, leading to the hypothesis that hypermethylation of the CpG islands was the mechanism responsible for decreasing ESR1 expression in tumor tissue. We subsequently

Table II. SNP signal at ESR1 gene locus.

Probe_Set_ID	Chromosome	Physical position	Normal cell	Confidence	Tumor cell	Confidence
SNP_A-4212066	6	152066213	AB	0.09375	AB	0.09375
SNP_A-4212068	6	152084195	AB	0.007813	AB	0.015625
SNP_A-2138703	6	152109562	AB	0.0625	AB	0.1875
SNP_A-2241225	6	152124339	AB	0.039063	AB	0.023438
SNP_A-4302034	6	152126286	AB	0.015625	AB	0.015625
SNP_A-2043771	6	152132228	AB	0.007813	AB	0.1875
SNP_A-2185504	6	152221934	AB	0.0625	AB	0.03125
SNP_A-1856863	6	152241822	AB	0.007813	AB	0.039063
SNP_A-2012176	6	152307215	AB	0.1875	AB	0.125
SNP_A-2131360	6	152349399	AB	0.007813	AB	0.007813
SNP_A-2032260	6	152350666	AB	0.007813	AB	0.007813
SNP_A-2182907	6	152370309	AB	0.09375	AB	0.015625
SNP_A-2126916	6	152389551	AB	0.039063	AB	0.09375
SNP_A-2055019	6	152413084	AB	0.1875	AB	0.078125
SNP_A-2021652	6	152414235	AB	0.0625	AB	0.039063
SNP_A-2166370	6	152418873	AB	0.007813	AB	0.007813
SNP_A-4233902	6	152424004	AB	0.0625	AB	0.125
SNP_A-4202504	6	152424018	AB	0.007813	AB	0.007813
SNP_A-1951801	6	152426493	AB	0.007813	AB	0.007813
SNP_A-2254273	6	152426929	AB	0.007813	AB	0.007813
SNP_A-2230180	6	152428321	AB	0.132813	AB	0.007813
SNP_A-1988124	6	152431055	AB	0.015625	AB	0.015625
SNP_A-4232738	6	152434853	AB	0.039063	AB	0.09375
SNP_A-4219387	6	152435454	AB	0.007813	AB	0.016602
SNP_A-2043027	6	152449819	AB	0.007813	AB	0.1875

Table III. Methylation array analysis of ESR1 gene.

Probe ID	Gene symbol	Sample	Methylation value	Total	Status		Confidence	Chromosomal location
					Unmethylated	Methylated		
cg00655307	ESR1	Normal	0.093132	3948	3571	377	3.68E-38	6q25.1
		Tumor	0.774897	3525	716	2809	3.68E-38	

confirmed hypermethylation by MSP in tumor tissue obtained from the 68-year-old woman (Fig. 1B).

MSP and UNMSP of nine cell lines. We designed primers for MSP and UNMSP and checked the methylation status of the HCC samples used for the arrays and nine HCC cell lines. We obtained bands of appropriate size in the lanes of tumor tissue for HuH2, HepG2, HLE, HLF and SK-Hep1 cells by electrophoresis of MSP. In UNMSP, however, there were bands in lanes of HuH1, HuH7, HepG2, Hep3B and PLC/PRF/5 cells (Fig. 1C). We consider that complete methylation existed in HuH2, HLE, HLF and SK-Hep1 cells, complete unmethylation in HuH1, HuH7, Hep3B and PLC/PRF/5 cells and partial methylation in HepG2 cells.

5-Aza-dC treatment of nine cell lines. The ESR1 expression of HuH2, HepG2, HLE, HLF and SK-Hep1 cells were reduced by promoter hypermethylation and were reactivated after 5-aza-dC treatment. HuH2, HLF and SK-Hep1 cells that underwent complete promoter hypermethylation showed increase in expression after 5-aza-dC treatment (Fig. 1D).

Sequence analysis. To confirm that the amplification of MSP was correctly performed, sequence analysis of the

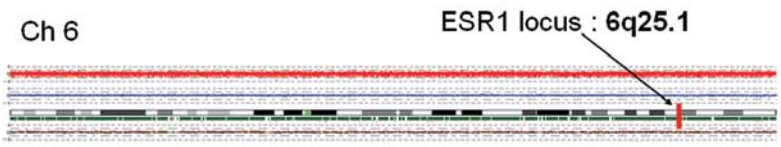

Figure 2. Copy number analysis of chromosome 6 by SNP array in HCC tissue. There was no deletion in copy number analysis using 500 K SNP array in HCC tissue. The ESR1 locus did not show any deletion or amplification.

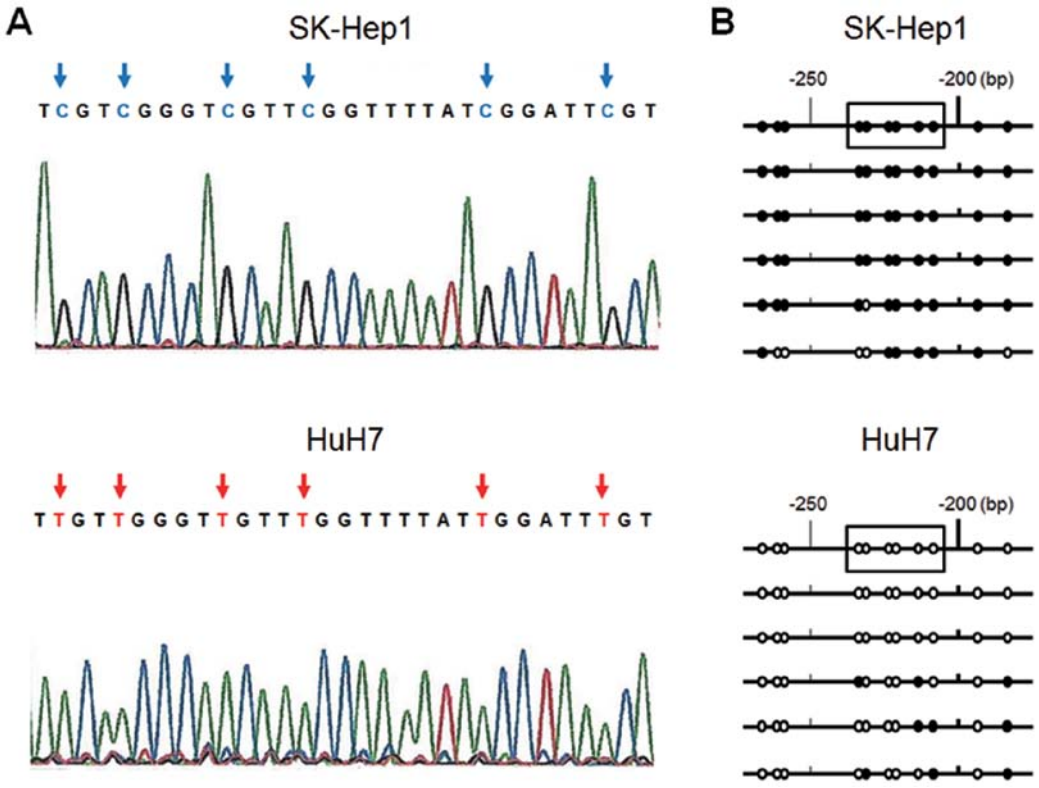

Figure 3. (A) Sequence analysis of bisulfite-treated DNA from HuH7 and SK-Hep1 cells localized to the ESR1 promoter region. Arrows indicate CpG islands. The C bases indicate methylated CpG islands in SK-Hep1 cells. The T bases were converted from C by bisulfite treatment, revealing unmethylated CpG islands in HuH7 cells. (B) Some of the six clones showed a different methylation status in HuH7 and SK-Hep1 cells. Closed circles represent methylated CpG islands; open circles indicate unmethylated CpG islands.

ESR1 promoter region was performed in six colonies by TA cloning in HuH7 and Sk-Hep1 cells. In most cases, we found all CpG islands in the fragment of SK-Hep1 cells to be CG, whereas all those of HuH7 cells were TG (Fig. 3A). Some of the six clones showed a different methylation status in both cell lines (Fig. 3B) and this result suggested that they were partially methylated and reflected the MSP and UMSP results.

MSP and UNMSP of samples from 48 HCC patients. A total of 40 (83.3%) of the 48 HCC tissues displayed promoter hypermethylation, but 32 (66.7%) of 48 normal tissues showed promoter hypermethylation of the ESR1 gene (Fig. 4A and B).

Quantitative PCR of samples from 48 HCC patients. Total ESR1 expression in HCC tissue was significantly lower than in normal tissue, relative to GAPDH (median ESR1/GAPDH ratio, 0.0175 vs. 0.0107; P<0.001) (Fig. 4C). In 24 (50%) of 48 HCC samples, the expression level of ESR1 gene decreased by >90% in HCC compared to normal tissue.

Correlation between the clinicopathological factors in 48 HCC patients and results of our findings. No correlation was found between promoter hypermethylation of ESR1 and patient clinicopathological factors in either normal or HCC tissue (data not shown). Disease-free and overall survival of patients with promoter hypermethylation of ESR1 in HCC tissue were not significantly shorter than in those without promoter hypermethylation of ESR1 (P=0.2378 and P=0.5402, respectively). On the other hand, In 24 (50%) HCC samples, the expression level of ESR1 gene was decreased by >90%. The decreased expression was significantly related to high liver damage score (P=0.0388), pathological invasion of the intrahepatic portal vein (P=0.0236), the size of tumor (>3 cm in diameter) (P=0.0417) and hepatitis B virus (HBV) infection (P=0.011). In contrast, disease-free survival and overall survival of patients with reduced ESR1 expression in HCC tissue were not significantly shorter than in those without reduced ESR1 expression in HCC tissue (P=0.4854 and P=0.4612, respectively).

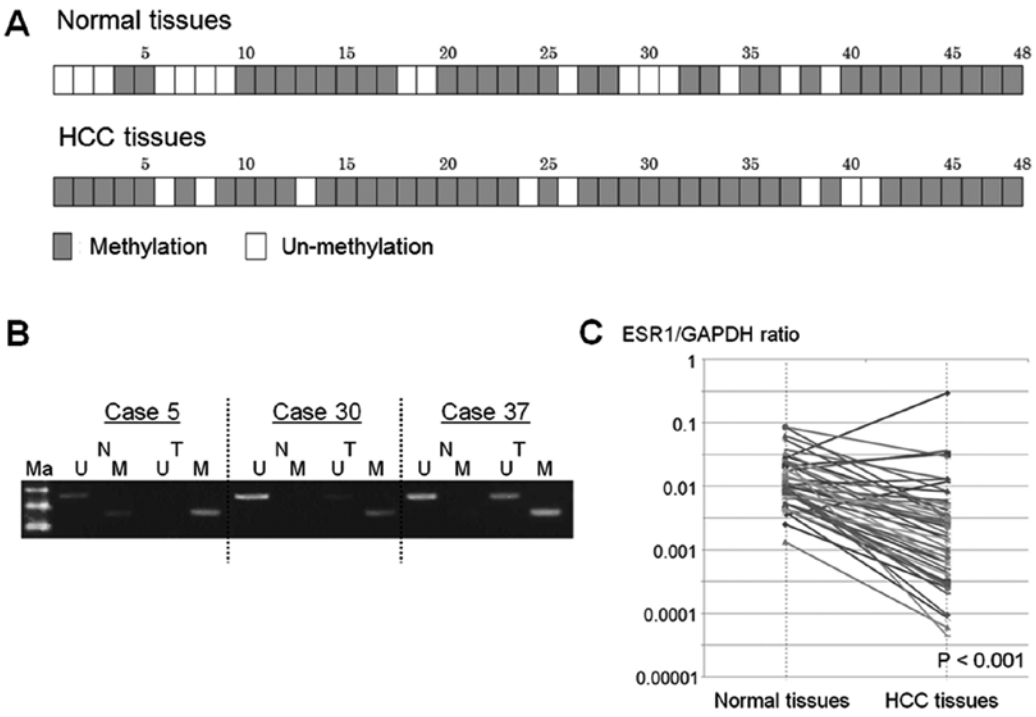

Figure 4. (A) Promoter hypermethylation of the ESR1 gene was detected in 40 (83.3%) of 48 tumor tissues. Gray squares indicate cases of promoter hypermethylation of the ESR1 gene, whereas white squares indicate no methylation of the ESR1 gene. (B) Three samples of paired normal and tumor tissue were chosen at random and analyzed for ESR1 methylation. These three cases showed promoter hypermethylation of tumor tissue. In case 5, there was little promoter hypermethylation of normal tissue. (C) Total ESR1 expression in tumor tissue was significantly lower compared with normal tissue (median ESR1/GAPDH ratio, 0.0175 vs. 0.0107; P<0.001). ESR1, estrogen receptor 1; Ma, marker; M, MSP; U, UNMSP; N, normal tissue; T, tumor tissue.

Discussion

Epidemiological reports indicate that the incidence of HCC is higher in male than in female patients (14). It has been observed that chronic liver disease progresses more rapidly to cirrhosis and HCC in male than female patients (15). Therefore, estrogens are considered to play an important role in liver diseases. Some studies report that estrogens have antifibrotic effects and are protective factors for the progression of fibrosis in patients with chronic hepatitis (16). Naugler *et al* have indicated in diethylnitrosamine-treated male mice that estrogen reduces circulating concentrations of interleukin (IL)-6, a proinflammatory molecule released from Kupffer cells upon exposure to necrotic hepatocytes. They have proposed that estrogen-mediated inhibition of IL-6 production reduces liver cancer risk (17). Estrogen has also been reported to suppress generation of oxidative-stress-induced reactive oxygen species (ROS), lipid peroxidation, activation of activator protein (AP)-1 and nuclear factor (NF)-κB, which are transcription factors (18).

We chose ESR1 gene as a tumor suppressor gene from the candidates, because of the above-mentioned functions of estrogens. ESR1 encodes estrogen receptor (ER)α, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding and activation of transcription. Furthermore, the relation between ESR1 and malignant disease has been discussed in a variety of tissues including breast, colon, blood, bladder and liver (19-27).

ERα plays key roles in cell development and differentiation. Many studies have investigated these roles in relation to breast cancer. In the presence of ligand, ERα may inhibit invasion through mechanisms involving transcriptional activation of estrogen response element regulating target genes, such as E-cadherin, which increases cell-cell adhesion (28). In the absence of ligand, ERα also inhibits invasion through a distinct mechanism involving protein-protein interaction with the region of the first zinc finger of ERα (28). It is considered that these roles of ERα are also applicable to HCC and result in a significant association between the decrease in ERα and pathological invasion of HCC.

In a recent Japanese nationwide investigation, the rate of HCV infection among the HCC patients was 72.3% and that of HBV infection was 16.8% (29). In the present study, the infection rates were compatible at 79.2 and 14.6%, respectively. The expression of ESR1 gene was decreased in all of the seven patients infected with HBV, implicating that it was inversely correlated with the HBV infection. Some reports actually discussed the relationship between ERs and HBV infection. The level of ERs in the cytosol of peripheral blood mononuclear cells is significantly lower in asymptomatic HBV carriers and patients with chronic hepatitis than in healthy controls (30,31). In contrast, estrogen can repress transcription of HBV genes by upregulating ERα, which interacts with and alters binding of hepatocyte nuclear factor-4α to HBV enhancer I (32). There is a possibility that the decrease in ERα (ESR1 expression) in liver tissue enhances the adverse influence of HBV infection and this may be the reason that the ESR1 expression was found in the present study to be lower in HCC than in normal tissue.

In this study, there were some cases that had hypermethylation of the promoter region in both non-cancer and HCC tissues. However, in the electrophoresis of MSP, the density

of the band in tumor tissue tended to be stronger than the band in non-cancer tissue. Therefore, it can be speculated that some promoter hypermethylated regions already existed in precancer liver tissue in the form of chronic hepatitis or cirrhosis and the hypermethylation status becomes further enhanced as these tissues develop into cancer.

The size of tumor with reduced ESR1 expression in HCC tissue was significantly larger than in those without reduced ESR1 expression in HCC tissue. Regarding the pathological diagnosis, however, the HCC with the reduced ESR1 expression tended to be well differentiated phenotype (P=0.1576) which is associated with more indolent biology. This could be the reason that the disease-free survival and overall survival of patients with the reduced ESR1 expression were not significantly shorter than in those without the reduced ESR1 expression.

In conclusion, our results indicate that ESR1 acts as a tumor suppressor gene and that one of the mechanisms of ESR1 silencing is related to promoter hypermethylation in human HCC. The novel method of triple-combination array analysis that was applied in this study is useful for detecting new suppressor oncogenes and their mechanisms and further investigations using this method is warranted.

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