Original Research

Methylation patterns of estrogen receptor α promoter correlate with estrogen receptor α expression and clinicopathological factors in hepatocellular carcinoma

Binghua Dai[1], Li Geng[1], Yong Yu[2], Chengjun Sui[1], Feng Xie[1], Weifeng Shen[1], Tao Zheng[1] and Jiamei Yang[1]

[1]The Department of Special Treatment, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China; [2]The Department of Biliary Tract Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

Corresponding author: Jiamei Yang. Email: jmyang@smmu.edu.cn

Abstract

Hepatocellular carcinoma (HCC) is the seventh most common type of cancer; notably, the incidence of HCC is four to eight times higher in men than women. Previous studies reported that the estrogen receptor (ER) signaling pathway is involved in the pathogenesis of HCC, although the extent of its involvement is unclear due to several conflicting reports. In the present study, tumor and paired adjacent non-cancerous tissues from 157 HCC patients were collected. Transcriptome sequencing and real-time quantitative polymerase chain reaction were used to quantify the ER α (ESR1) expression levels, and the Sequenom EpiTYPER assay was used to delineate the methylation patterns in the ESR1 promoter. We found that ESR1 expression was significantly reduced in tumor tissues ($P < 0.001$) compared to adjacent non-cancerous tissues. The CpG sites around the transcription start site were significantly hypermethylated in the tumor ($P < 0.0001$). This methylation pattern also correlated with the gene expression ($P < 0.0001$). Additionally, we found that the hypermethylation of ESR1 was associated with the presence of fibrous capsules ($P = 1.2 \times 10^{-4}$), the absence of microvascular invasions ($P = 8.0 \times 10^{-4}$), thin trabecular pattern ($P = 0.025$), and lower histologic gradings ($P = 5.2 \times 10^{-3}$). Thus, ESR1 expression is a candidate tumor suppressor gene in HCC. Further, promoter hypermethylation may be a mechanism by which expression of ESR1 is repressed, and the extent of hypermethylation of ESR1 may be a marker for HCC status and progression.

Keywords: Estrogen receptor α, hepatocellular carcinoma, methylation

***Experimental Biology and Medicine* 2014; 239: 883–890. DOI: 10.1177/1535370214536651**

Introduction

Hepatocellular carcinoma (HCC) is the seventh most common type of cancer and the third leading cause of cancer-related deaths in the world.[1] In China, it is estimated that there are more than 300,000 new HCC cases diagnosed annually.[2] HCC is sexually dimorphic in humans, with a significantly higher incidence in males; male-to-female ratios average between 2:1 and >4:1 across studies, especially in the presence of hepatitis B (HBV)-related HCC, which is five- to sevenfold higher in male HBV carriers than in female carriers.[3] In addition, the unbalanced relationship with gender is not limited to incidence; prognosis is also better in females, including spontaneous survival and survival after resection. It is possible that sex hormones may play a role in modulating HCC risk or development.[4]

The liver is a sexually dimorphic organ, with gender differences in gene expression, mitochondrial function, microsomal enzyme activity, membrane lipid composition, and immune responses. Sex hormones are considered to play an important role in regulating liver function and morphology, especially estrogen. In fact, estrogens are involved in the regulation of hepatocyte proliferation. In the liver, experimental models have shown that estrogens act as tumor promoters and may induce hepatocarcinogenesis in hamsters and mice;[5] estrogens may also induce the formation of free radical-mediated DNA and RNA adducts which are potentially mutagenic. In humans, the chronic use of estrogens is associated with increased risk of developing liver neoplasms such as benign nodular hyperplasia and hepatic adenoma.[6] According to this, it seems that HCC would be more common in women because women are

Copyright © 2014 by the Society for Experimental Biology and Medicine

exposed to more estrogen than men. However, this is contradictory to the fact that HCC has a higher incidence in men.

Estrogen-stimulated growth requires two estrogen receptors (ERs), α and β, which are members of the superfamily of nuclear receptors. The ERs in primary liver tumors have been experimentally studied over recent decades.[5,7,8] Both isoforms of ERs (ER α and ER β) have been reported to be variably expressed in HCC.[9] However, the role of ERs in HCC is not fully understood, and the previously reported ER α expression changes have not been consistently observed in subsequent studies. For example, one study reported an association between increased expression of the ERs and estrogen-induced tumor formation in ESR1-mutated transgenic mice, which overexpress ESR1 and are highly sensitive to the carcinogenic effects of synthetic estrogens.[10] On the other hand, several studies have found that decreased ER α (ESR1) expression in liver tissue enhances the adverse effects of HBV infection, and ESR1 was defined as a tumor suppressor gene.[11–13] In this study, we address the role of ESR1 in HCC by using transcriptome sequencing and real-time quantitative polymerase chain reaction (PCR) to identify the ESR1 expression changes in HCC.

In addition, the ESR1 promoter contains several CG-rich regions (CpG islands) proximal to the transcription start site (TSS) of the ESR1 gene, so we hypothesized that methylation of promoter CpG islands may be an important mechanism of ESR1 gene inactivation. Previous studies in breast cancer cell lines and tumors have also confirmed the ESR1 expression was associated with aberrant CpG island hypermethylation. We, therefore, used the Sequenom EpiTYPER assay to study the relationship between the methylation changes, ESR1 expression, HCC, and its clinical characteristics, especially for some important clinical pathological factors in the HCC progression, such as fibrous capsule, microvascular invasion, architectural patterns, and histologic grading.

Materials and methods

Patients and tissue samples

HCC tissues and corresponding adjacent non-cancerous tissues were obtained from 157 patients treated at the Shanghai Eastern Hepatobiliary Surgery Hospital between January 2012 and December 2012. Their ages ranged from 22 to 76 years (51.7 ± 10.3 years: means ± standard deviation), and the male-to-female ratio was 133:24.

Serum alphafetoprotein (AFP), carcinoembryonic antigen (CEA), and CA19-9 were measured by the electrochemiluminescence immunoassay on cobas E601 immunoassay analyzers (Roche Diagnostics, GmbH, Mannheim, Germany).

All tissues collected were diagnosed pathologically as HCC. The diagnosis of the HCC was based on morphology and immunohistochemistry as suggested by WHO,[14] and the immunohistochemistry tests were performed on 4% formaldehyde-fixed tissues. Morphologically, HCCs consist of tumor cells that usually resemble hepatocytes. Nuclei are usually prominent with prominent nucleoli and a high nuclear:cytoplasmic ratio, and there is usually some hyperchromatism and nuclear irregularity, although this is quite variable. The tumour cells usually have distinct cell membranes and a moderate amount of eosinophilic, finely granular cytoplasm. Immunohistochemically, hepatocyte paraffin 1, glypican 3, CD10, and polyclonal antiserum to carcinoembryonic antigen (pCEA) were used to differentiate HCC from other liver tumors.

Grading of the HCCs was based on the Edmondson–Steiner grading system,[15] a scale ranging from grade 1 to grade 4, on the basis of nuclear and cellular atypia. Tumor sizes were defined as the largest dimension for solitary masses and the size of the largest tumor for multiple masses.

The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. All of the samples and associated clinical information were collected with informed consent of patients, and all of the experiments were approved by the Internal Review and Ethics Boards of the Shanghai Eastern Hepatobiliary Surgery Hospital.

DNA/RNA extraction

Genomic DNA was isolated from 25 μg tissue samples using the QIAamp DNA Mini Kit following the manufacturer's protocol (QIAGEN, Hilden, Germany).

RNA was extracted from fresh, frozen tissue using RNA isolation reagents (TRIzol; Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Takara).

cDNA synthesis was performed using SuperScript® III First-Strand Synthesis Kits according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Transcriptome sequencing and data analysis

Total RNA (4 μg) from five patients were used for transcriptome library preparation, according to the standard procedure of the TruSeq RNA SamplePrep Guide v2 (Illumina). Sequencing was performed on the Illumina Hiseq2000 sequencing system. Raw RNA-seq data was filtered by Trimmomatic-0.20[16] according to the following criteria: (1) reads containing sequencing adaptors were removed; (2) nucleotides with a quality score lower than 20 were removed; and (3) reads shorter than 36 were discarded. The clean reads were then aligned with the UCSC mm9 reference genome using TopHat v2.0.3,[17] which calls Bowtie v0.12.8 to perform the alignment.[18] The prebuilt index of UCSC hg19 was downloaded from the TopHat home page (http://tophat.cbcb.umd.edu/index.html) and used as the reference genome. TopHat first splits reads into shorter segments, and then Bowtie reports successfully mapped segments which allow a maximum of two mismatches. TopHat allows multiple alignments per read (up to 20 by default) when mapping the reads to the reference. The default parameters for the TopHat method were used. The alignment files produced by TopHat were supplied to Cuffdiff v2.0.2[19] along with the downloaded UCSC GTF file. Cuffdiff re-estimates the abundance of the transcripts listed in the GTF file using alignments from the BAM file and concurrently tests for differential expression. The normalized expression level of each gene was measured by

fragments per kilobase per million (FPKM), including ESR1 gene.

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI Prism 7900 sequence detection system using Power SYBR Green (Applied Biosystems). The primers used for real-time PCR are mainly from PrimerBank (http://pga.mgh.harvard.edu/primerbank). For ESR1, forward primer, 5′-CCCACTCAACAGCGTGTCTC-3′ and reverse primer, 5′-CGTCGATTATCTGAATTTGGCCT-3′; for GAPDH, forward primer, 5′-GGAGCGAGATCCCTCCAAAAT-3′ and reverse primer, 5′-GGCTGTTGTCATACTTCTCATGG-3′. The PCR reactions included 1 × Sybr green Taqman Gene Expression master mix (Applied Biosystems), 10 nM forward and reverse primer and 5 μL 20-times diluted cDNA template. Cycling conditions for all primer pairs were 95°C incubation for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For quality control, all reactions were performed in duplicate and checked for concordance to capture intraassay variability. The expression of GAPDH was used to normalize that of the ESR1gene.The average was calculated according to the triplicate assay, and the expression level of ESR1 was expressed as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct$ (ESR1)-Ct (GAPDH).

Genomic DNA bisulfite conversion and Sequenom analysis

Genomic DNA (500 ng) was modified by sodium bisulfite treatment according to the protocol of the EpiTect Fast DNA Bisulfite Kit (QIAGEN, Hilden, Germany). The DNA was then used in the Sequenom EpiTYPER assay.

The ESR1 forward aggaagagagATTTGTTAAAGG TGGTTTAGGGAAG and reverse cagtaatacgactcactataggg agaaggctAATCCAACATAAACACACAAATCAA primers were used for Sequenom methylation analysis. The amplicon comprised 12 CpG sites located in Human Genome 19 assembly – chr6:152,128,095-152,128,580 (GRCh37/hg19) (see Supplementary Figure 1 and Supplementary Table 1). Through PCR amplification, SAP cleanup, T Cleavage, and conditioning of the hMC reaction products with Clean Resin steps, the products were transferred to a SpectroCHIP® array and analyzed on the MassARRAY® Analyzer 4 instrument. In addition, 24 DNA samples from 12 patients were randomly selected to be replicated in this study and yielded a highly consistent result ($R^2 = 0.95$).

Data analysis

The statistical analyses were performed using the SPSS (SPSS, Chicago, IL, USA) program. A P value <0.05 was considered significant. The ESR1 expression and methylation changes were compared by using paired t-test between primary tumors and adjacent non-cancerous tissues. The association of methylation between methylation changes and clinical parameters were detected by using linear regression analysis.

Results

Population characteristics

The clinical characteristics of the patients are summarized in Table 1. Of the 157 patients, 133 patients had HBV (male-to-female ratio = 112:21) and four patients had hepatitis C. Forty-seven patients had an alcohol habit (more than 50 g/day), and 15 patients had a family history of HCC. For the clinical characteristics, 14 patients had more than one primary tumor and 26 patients presented with satellite tumors; macrovascular invasions were detected in

Table 1 The clinical characteristics of the HCC patients in this study

Characters	Type	No.
Sex	Female	24
	Male	133
Hepatitis	None	20
	HBV	133
	HCV	4
Alcohol habit(≥ 50 g/day)	No	110
	Yes	47
High blood pressure	No	143
	Yes	14
Type 2 diabetes	No	144
	Yes	13
Family history	No	142
	Yes	15
Age (mean ± SD)	51.7 ± 10.3	
AFP (mean ± SE)	29072.2 ± 15452.0	
CEA (mean ± SD)	2.84 ± 1.71	
CA19-9 (mean ± SE)	32.1 ± 5.74	
Diameter of tumor (mean ± SD)	6.33 ± 3.63	
Fibrous capsule	No	44
	Yes	113
Satellite tumor	No	131
	Yes	26
Macrovascular invasion	No	151
	Yes	6
Microvascular invasion	No	113
	Yes	44
Number of tumor	1	143
	2	9
	3	5
Liver cirrhosis	None	108
	Micronodular cirrhosis	28
	Mixed macro-micro-nodular cirrhosis	19
	Macronodular cirrhosis	2
Architectural patterns	Thin trabecular pattern	16
	Thicker trabecular pattern	112
	Other types	17
Histologic grading	<III grade	12
	III grade	139
	>III grade	6

HCC: hepatocellular carcinoma; AFP: alphafetoprotein; CEA: carcinoembryonic antigen.

six patients and microvascular invasions in 44 patients; 108 patients had no liver cirrhosis and 139 patients were classified with grade III cirrhosis.

ESR1 expression is decreased in tumor tissue

To examine whether ESR1 expression changes in HCC, whole transcriptome sequencing was used in five selected patients (HBV positive and male). From the FPKM value of ESR1 gene, we identified that the ESR1 expression was significantly reduced in all five tumor tissues $P = 0.0013$) (Figure 1(a)), and the expression level of ESR1 gene decreased by >90% in HCC compared to adjacent non-cancerous tissues.

In addition, we downloaded the transcriptome sequencing data from TCGA (http://cancergenome.nih.gov/), which comprise 69 tumor tissues and 36 normal tissues The FPKM value of ESR1 gene in this data also showed a decreased ESR1 expression in tumor tissues ($P < 0.0001$) (Figure 1(b)).

Consequently, we used quantitative real-time PCR to validate the decreased expression changes in 35 patients.

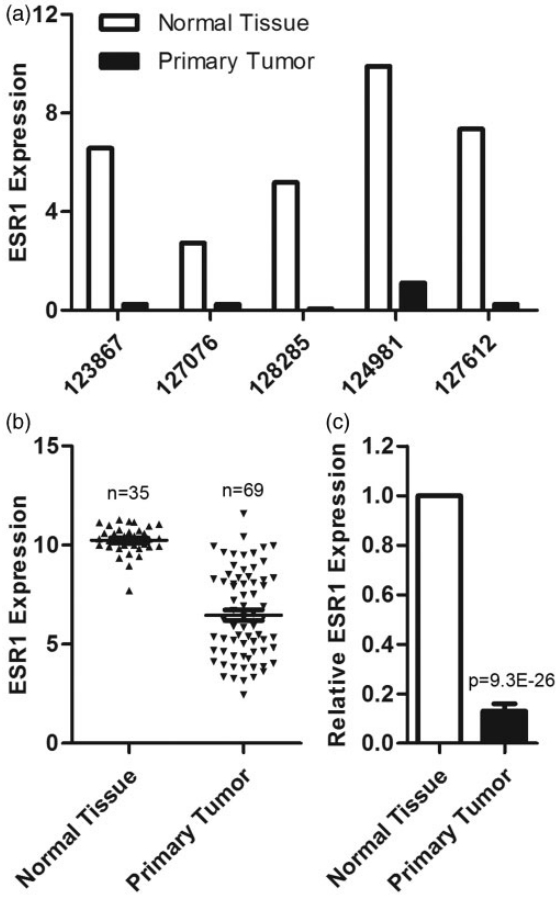

Figure 1 ESR1 expression levels between tumor and normal tissues in HCC. Transcriptome sequencing in five patients indicated the ESR1 expression was largely decreased in tumor tissues (a) and the ESR1 expression were significantly reduced according to the data from TCGA project (http://cancergenome.nih.gov/) (b) Real-time quantitative PCR in 35 patients further confirmed the decreased ESR1 expression in tumor tissues.
ESR1: estrogen receptor α

Total ESR1 expression in all 35 HCC tissues was significantly lower than in adjacent non-cancerous tissues ($P < 0.0001$) (Figure 1(c)).

The ESR1 gene promoter is hypermethylated in tumor tissue

For the methylation status of the ESR1 promoter, a 486-bp amplicon including 12 CpG sites were designed, and only six CpGs (CpG4, CpG5, CpG6, CpG7, CpG10, and CpG12) were successfully genotyped by using Sequenom Technology (see Supplementary Figure 1). First, we analyzed the correlation among the six CpGs sites, and they were all significantly correlated with each other (see Supplementary Table 2). Then, we analyzed the correlation among the methylation of the six CpGs sites in adjacent non-cancerous tissues, the methylation of the six CpGs sites in tumor tissues, and the difference of six CpGs sites between paired tissues; the data indicated that the methylations of the six CpGs sites in tumor were not correlated to the methylation of these sites in adjacent non-cancerous tissues (see Supplementary Table 3), but the differences between paired tissues were correlated with the methylation of the six CpGs sites in both tumor and adjacent non-cancerous tissues (see Supplementary Table 3).

Methylation levels of the six CpG sites in ESR1 promotor were pair analyzed, and five CpG sites (CpG4, CpG5, CpG6, CpG7, and CpG10) showed significant differences in methylation status between tumor and adjacent non-cancerous tissues. The mean differences are summarized in Table 2, with the major findings being that the tumor tissues were significantly hypermethylated ($P < 0.0001$), and that there was more than 10% in the difference in levels of methylation between paired tissues (from 12% to 25%).

The correlation between methylation and ESR1 expression

In this study, the methylation and gene expression were both investigated in 35 paired tissues of patients, so we performed a correlation analysis between methylation status and expression, and the results indicated that the ESR1 expression was significantly negatively associated with the CpG sites' (CpG4, CpG5, CpG6, CpG7, and CpG10) methylation in the ESR1 promoter ($P < 0.0001$, 4.2×10^{-4}, 4.2×10^{-4}, 1.3×10^{-3}, and 2.0×10^{-4}, respectively; Table 3), excluding the CpG12 site. Moreover, the mean methylations were also correlated with ESR1 expression (including and excluding the CpG12 site, $P < 0.0001$ and 1.5×10^{-4}) (Table 3).

The association between ESR1 methylation and clinical pathological factors

Statistical analysis of the correlation between the six CpG sites' methylation levels and clinical pathological factors was performed in four groups of patients (including all 157 HCC patients, 133 male HCC patients, 133 HCC patients with HBV, and 112 male HCC patients with HBV). The evaluated categories were age, alcohol habit, high blood pressure, type 2 diabetes, family history, Age,

Table 2 The difference between the methylation patterns of the six CpG sites in paired tissues

			HCC ($N = 158$)			HCC-Male ($N = 133$)			HCC-HBV ($N = 133$)			HCC-Male-HBV ($N = 112$)		
Position	**CpGs**	**Group**	**Mean**	**ΔMean**	***P* Value**	**Mean**	**ΔMean**	***P* Value**	**Mean**	**ΔMean**	***P* Value**	**Mean**	**ΔMean**	***P* Value**
Chr3:152128515	CpG4	Normal	31.1%	−23.0%	<0.0001	30.6%	−24.1%	<0.0001	30.8%	−23.4%	<0.0001	30.5%	−25.0%	<0.0001
		Tumor	54.1%			54.8%			54.3%			55.5%		
Chr3:152128483	CpG5	Normal	27.5%	−17.6%	<0.0001	27.5%	−18.3%	<0.0001	27.2%	−18.1%	<0.0001	27.1%	−19.6%	<0.0001
		Tumor	45.1%			45.8%			45.3%			46.7%		
Chr3:152128471	CpG6	Normal	27.5%	−12.0%	<0.0001	27.7%	−12.4%	<0.0001	27.4%	−12.3%	<0.0001	27.5%	−13.3%	<0.0001
		Tumor	39.5%			40.1%			39.7%			40.8%		
Chr3:152128426	CpG7	Normal	30.1%	−12.4%	<0.0001	30.5%	−12.1%	<0.0001	30.3%	−12.1%	<0.0001	30.7%	−12.5%	<0.0001
		Tumor	42.5%			42.6%			42.4%			43.2%		
Chr3:152128338	CpG10	Normal	33.1%	−19.0%	<0.0001	34.2%	−19.0%	<0.0001	32.8%	−20.1%	<0.0001	34.1%	−20.6%	<0.0001
		Tumor	52.1%			53.2%			52.9%			54.7%		
Chr3:152128258	CpG12	Normal	47.8%	1.4%	0.622	46.7%	−0.2%	0.941	47.0%	0.1%	0.964	45.6%	−2.5%	0.471
		Tumor	46.4%			46.9%			46.8%			48.1%		

HCC: hepatocellular carcinoma; HBV: hepatitis B.

AFP, CEA, CA19, diameter of tumor, fibrous capsule, satellite tumor, macrovascular invasion, microvascular invasion, number of tumors, liver cirrhosis, architectural patterns, and histologic grading. In all four groups, we found that the methylation levels of ESR1 were associated with the presence of fibrous capsules, microvascular invasion, architectural patterns, and histologic grading (Table 4, Supplementary Tables 4 to 7). For the mean methylation levels of the six CpG sites in tumor tissues in all HCC patients (Table 4), hypermethylation was found to be associated with the presence of fibrous capsules ($P = 1.2 \times 10^{-4}$), the absence of microvascular invasion ($P = 8.0 \times 10^{-4}$), a thin trabecular pattern ($P = 0.025$), and a lower histologic grading ($P = 5.2 \times 10^{-3}$). For the mean difference of methylation between paired tissues in all HCC patients (Table 4), bigger methylation changes were found to be correlated with the presence of fibrous capsules ($P = 1.2 \times 10^{-3}$), the absence of microvascular invasion ($P < 0.0001$), a thin trabecular pattern ($P = 0.021$), and a lower histologic grading ($P = 3.5 \times 10^{-3}$).

Discussion

Previous epidemiological studies indicated that the incidence of HCC is higher in male than in female patients,[3] and subsequent studies showed that the estrogen and ER signaling pathways were involved in the pathogenesis of HCC.[20] ESR1 encodes ER α, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. Moreover, ESR1 has been found to be a key molecule in several sex-related cancers, including breast,[21] bladder,[22] and ovarian.[23] Here, by using transcriptome sequencing and real-time quantitative PCR, we consistently found that the ESR1 expression was largely decreased in tumor tissues compared with adjacent non-cancerous tissues, indicating that suppression of ER signaling pathways represents an important change for tumorigenesis in HCC. The data downloaded from TCGA also confirmed the reduced ESR1 expression in HCC. However, we also observed that ESR1 expression was not consistently depressed in American HCC patients, and that there was a smaller difference of mean expression between tumor and normal tissues in this group. We thought that the inverse relationship between ERs and HBV infections might be one reason for these differences among the different studies. Previously, Mizoguchi et al.[24,25] reported that 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. In contrast, Wang et al.[12] found that estrogen can repress transcription of HBV genes by upregulating ER α, which interacts with, and alters binding of hepatocyte nuclear factor-4α to HBV enhancer I. Approximately 5% of the world population (350–400 million people) is chronically infected with HBV; 75% of infected people are Asian, in contrast with a lower prevalence of HBV infection (0.3%–1.5%) in western countries.[1] In our study, 84.7% patients (133:24) have HBV infections. This helps to explain why ESR1 expression was so strongly decreased in cancerous tissue, relative to

Table 3 The correlation of the methylation status of the six genotyped CpG sites and ESR1 expression

Correlations		R^2 CpG4	CpG5	CpG6	CpG7	CpG10	CpG12	ΔMeanB*	ΔMeanB†	ESR1 expression
P value	CpG4	1	0.86	0.89	0.73	0.94	0.46	0.96	0.93	**−0.52**
	CpG5	7.6 E-21	1	0.92	0.84	0.88	0.65	0.94	0.95	**−0.42**
	CpG6	5.0 E-24	2.9 E-28	1	0.76	0.93	0.61	0.94	0.94	**−0.42**
	CpG7	1.9 E-12	2.8 E-19	3.9 E-14	1	0.74	0.65	0.84	0.86	**−0.38**
	CpG10	2.1 E-33	1.4 E-23	1.1 E-29	3.5 E-13	1	0.56	0.96	0.95	**−0.44**
	CpG12	8.3 E-05	1.8 E-09	3.3 E-08	1.7 E-09	6.8 E-07	1	0.58	0.68	**−0.06**
	ΔMeanB*	1.8 E-37	3.4 E-32	1.2 E-32	3.2 E-19	9.5 E-39	1.7 E-07	1	0.99	**−0.48**
	ΔMeanB†	3.5 E-31	3.3 E-34	4.3 E-33	7.9 E-21	4.5 E-36	1.3 E-10	1.8 E-60	1	**−0.44**
	ESR1 expression	<0.0001	**4.2 E-04**	**4.2 E-04**	**1.3 E-03**	**2.0 E-04**	**0.63**	<0.0001	**1.5 E-04**	1

ESR1: estrogen receptor α.
*excluding CpG12 site.
†including CpG12 site.

non-cancerous control tissue. Moreover, these results suggest that ESR1 might play a more important role in HCC tumorigenesis in the Chinese population compared to other populations, especially European and American populations.

Alterations in DNA methylation frequently occur in hepatocellular cancer.[26,27] Promoter hypermethylation can lead to epigenetic inactivation of tumor-related genes, which plays a pivotal role in tumorigenesis. In this study, we found that the ESR1 promoter was hypermethylated in the tumor tissue of hepatocellular cancer, and that these methylation levels were correlated with the gene expression. These findings indicate that epigenetic regulation of the ESR1 promoter via hypermethylation may be one of the critical mechanisms for depression of this gene in HCC, and the ESR1 could be considered as the candidate suppressor gene in HCC.

In the present study, there are three reasons for the amplicon region which we selected to be genotyped for methylation study. First, the ESR1 gene has been reported to have eight promoter regions (A, B, C, D, E1, E2, T1&T2, and F) in the human genome, the gene uses different promoter region in different tissues and cells,[28] and the amplicon selected in the present study was located in the promoter B region. Second, the amplicon region coincided with the CpG island of the ESR1gene. Third, the TSS is located in this amplicon, just between the CpG6 and CpG7. Cumulatively, these observations suggest that this amplicon region could be a very important regulation box of the ESR1 gene.

To our knowledge, fibrous capsules, microvascular invasion, architectural patterns, and histologic grading are important clinical pathological factors in the HCC progression. Here, we also demonstrated that the methylation levels of the ESR1 promoter were associated with fibrous capsules, microvascular invasion, architectural patterns, and histologic grading in HCC. Hypermethylation was also observed to be associated with the presence of fibrous capsules, the absence of microvascular invasion, thin trabecular pattern, and lower histologic grading. Generally, loss of fibrous capsules, the presence of microvascular invasion, a thick trabecular pattern, and a higher histologic grading indicate worse prognosis and survival in HCC patients. So these results seem to be contrary to the tumor suppressor function of the ESR1 gene, as considered in previous studies.[29] We thought there were two reasons: first, ESR1 is not just a ligand-activated transcription factor. ESR1 expression was regulated by some common factors, such as estrogen,[4] p53 binding,[30–32] and microRNA-18 a.[11] Sometimes, growth factors could activate protein-kinase cascades and lead to phosphorylation and activation of nuclear ERs at EREs though ligand-independent genomic actions. So in a different microenvironment, the ESR1 might be involved in more pathways, and so more research is needed to study the function of ESR1 in HCC; second, a similar splice variant (vER α) has been described as being associated with tumor pathogenesis,[33] and the presence of the vER receptor can influence the natural history of patients with HCC. The presence of the liver vER α transcript in the tumor has been described as the strongest negative predictor of survival in operable HCC patients.[34] Furthermore, the presence of vER α was found to correlate with a higher clinical aggressiveness of the tumor in comparison with the tumors characterized by wild-type ER α transcript. So the more aggressive tumor might express more vER α, and the ESR1 promoter might be also further demethylated in aggressive HCC. Thus, the methylation levels of ESR1 might be a marker for HCC status and progression, and the ESR1 was further identified to be the candidate suppressor gene in HCC.

In summary, ESR1 expression was largely decreased in tumor tissues in HCC, and hypermethylation of the ESR1 promoter could be one of the mechanisms for depression of this gene in HCC. The methylation levels of ESR1 were also correlated with the presence of fibrous capsules, microvascular invasion, architectural patterns, and increased histologic grading in HCC. Thus, the methylation levels of ESR1 might be a marker for HCC status and progression.

Table 4 The association between the methylation and clinicopathological factors

Site	Tissues	Fibrous capsule	HCC			Microvascular invasion	HCC			Histologic grading*	HCC			Architectural patterns†	HCC		
			N	Mean	Sig.		*N*	Mean	Sig.		*N*	Mean	Sig.		*N*	Mean	Sig.
C-CpG4	Normal tissue	No	42	31.2%	0.904	No	95	29.2%	0.042	0	11	29.5%	0.732	1	13	27.9%	0.395
		Yes	93	30.8%		Yes	40	35.0%		1	124	31.1%		2	112	31.7%	
C-CpG5		No	42	28.9%	0.388	No	97	25.8%	0.029	0	11	25.9%	0.710	1	13	24.7%	0.426
		Yes	95	26.7%		Yes	40	31.3%		1	126	27.5%		2	114	27.9%	
C-CpG6		No	42	27.7%	0.869	No	97	26.6%	0.161	0	11	25.5%	0.567	1	13	24.5%	0.280
		Yes	95	27.4%		Yes	40	29.6%		1	126	27.6%		2	114	28.2%	
C-CpG7		No	42	28.4%	0.562	No	97	28.5%	0.199	0	11	30.9%	0.786	1	13	28.0%	0.633
		Yes	95	30.2%		Yes	40	32.4%		1	126	29.5%		2	114	30.2%	
C-CpG10		No	42	33.9%	0.679	No	97	32.1%	0.318	0	11	32.8%	0.967	1	13	31.3%	0.659
		Yes	95	32.6%		Yes	40	35.2%		1	126	33.0%		2	114	33.4%	
C-CpG12		No	42	50.0%	0.395	No	97	47.2%	0.453	0	11	49.4%	0.793	1	13	50.1%	0.787
		Yes	95	47.1%		Yes	40	49.8%		1	126	47.8%		2	114	48.7%	
T-CpG4	Primary tumor	No	43	42.4%	2.8 E-04	No	105	59.4%	7.6 E-04	0	12	65.0%	0.162	1	16	67.4%	0.039
		Yes	106	59.6%		Yes	44	43.4%		1	137	53.7%		2	123	52.7%	
T-CpG5		No	43	35.3%	9.5 E-04	No	105	50.6%	1.2 E-03	0	12	63.1%	0.019	1	16	62.8%	5.2 E-03
		Yes	106	50.7%		Yes	44	35.6%		1	137	44.7%		2	123	43.3%	
T-CpG6		No	43	30.1%	1.1 E-04	No	105	43.4%	1.1 E-03	0	12	49.9%	0.074	1	16	50.9%	0.018
		Yes	106	43.9%		Yes	44	31.8%		1	137	39.1%		2	123	38.2%	
T-CpG7		No	43	33.1%	8.7 E-04	No	104	44.6%	0.105	0	12	54.8%	0.050	1	16	53.3%	0.033
		Yes	105	46.5%		Yes	44	38.0%		1	136	41.5%		2	122	40.5%	
T-CpG10		No	43	39.8%	<0.0001	No	105	58.2%	2.5 E-04	0	12	67.5%	0.052	1	16	68.9%	0.012
		Yes	106	58.5%		Yes	44	40.9%		1	137	51.8%		2	123	50.7%	
T-CpG12		No	43	37.4%	3.5 E-03	No	105	52.1%	3.3 E-03	0	12	69.8%	3.9 E-03	1	16	69.6%	5.4 E-04
		Yes	106	52.0%		Yes	44	37.5%		1	137	45.8%		2	123	44.0%	
ΔCpG4	Difference	No	42	−11.6%	3.8 E-03	No	92	−30.3%	<0.0001	0	11	−36.8%	0.127	1	13	−40.9%	0.028
		Yes	90	−28.3%		Yes	40	−6.3%		1	121	−21.8%		2	110	−20.5%	
ΔCpG5		No	42	−6.8%	3.4 E-03	No	94	−24.2%	<0.0001	0	11	−37.7%	0.016	1	13	−39.0%	4.6 E-03
		Yes	92	−22.5%		Yes	40	−2.0%		1	123	−15.8%		2	112	−14.5%	
ΔCpG6		No	42	−2.9%	1.0 E-03	No	94	−16.9%	<0.0001	0	11	−26.7%	0.021	1	13	−29.2%	2.3 E-03
		Yes	92	−16.2%		Yes	40	−0.5%		1	123	−10.7%		2	112	−9.5%	
ΔCpG7		No	42	−5.1%	0.030	No	93	−16.0%	0.016	0	11	−28.8%	0.031	1	13	−29.4%	9.5 E-03
		Yes	91	−15.8%		Yes	40	−4.1%		1	122	−10.9%		2	111	−9.4%	
ΔCpG10		No	42	−6.3%	8.8 E-04	No	94	−25.6%	<0.0001	0	11	−32.5%	0.122	1	13	−36.6%	0.026
		Yes	92	−24.8%		Yes	40	−3.6%		1	123	−17.8%		2	112	−16.6%	
ΔCpG12		No	42	12.4%	9.4 E−03	No	94	−4.1%	3.0 E-03	0	11	−20.9%	0.019	1	13	−19.8%	9.1 E-03
		Yes	92	−3.6%		Yes	40	14.4%		1	123	3.4%		2	112	5.5%	
ΔMean		No	42	−3.4%	1.2 E-03	No	94	−19.4%	<0.0001	0	11	−30.6%	0.021	1	13	−32.5%	3.5 E-03
		Yes	92	−18.4%		Yes	40	−0.3%		1	123	−12.2%		2	112	−10.8%	

HCC: hepatocellular carcinoma.

*0: < III grade; 1: III grade and >III grade.

†1: Thin trabecular pattern; 2: Thicker trabecular pattern and other types

Author contributions: BD, LG, and YY contributed equally to this work. JY designed the whole study and supervised the experiments and data analysis. BD and JY supervised patient diagnosis and sample recruitment. BD, LG, and YY conducted data analyses, drafted the manuscript and performed or contributed to the main experiments. YY, CS, FX, WS, and TZ recruited samples. All authors critically reviewed the manuscript and approved the final version.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (81172304); the Science Foundation of Shanghai Municipal Public Health Bureau (2010127).

REFERENCES

1. El-Serag H. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012;**142**:1264–73
2. Yang JD, Roberts LR. Hepatocellular carcinoma: a global view. *Nat Rev Gastroenterol Hepatol* 2010;**7**:448–58
3. El-Serag H, Rudolph K. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007;**132**:2557–76
4. Erica V. Role of estrogen in liver cancer. *Womens Health (Lond Engl)* 2008;**4**:41–50
5. Maria N, Manno M, Villa E. Sex hormones and liver cancer. *Mol Cell Endocrinol* 2002;**193**(1–2):59–63
6. Christopherson WM, Mays ET, Barrows GH. Liver tumors in women on contraceptive steroids. *Obstet Gynecol* 1975;**46**:221–3
7. Naofumi N, Hirofumi Y, Yuichiro O, Akio I. Estrogen receptors in hepatocellular carcinoma. *Cancer* 1986;**57**:87–91
8. Shimizu I. Impact of oestrogens on the progression of liver disease. *Liver Int* 2003;**23**:63–9
9. Boix L, Bruix J, Castells A, Fuster J, Bru C, Visa J, Rivera F, Rodes J. Sex hormone receptors in hepatocellular carcinoma. Is there a rationale for hormonal treatment? *J Hepatol* 1993;**17**:187–91
10. Couse JF, Davis VL, Hanson RB, Jefferson WN, McLachlan JA, Bullock BC, Newbold RR, Korach KS. Accelerated onset of uterine tumors in transgenic mice with aberrant expression of the estrogen receptor after neonatal exposure to diethylstilbestrol. *Mol Carcinog* 1997;**19**:236–42
11. Wan–Hsin L, Shiou–Hwei Y, Cho–Chun L, Sung–Liang Y, Hsuan–Yu C, Chien–Yu L, Ding–Shinn C, Pei–Jer C. MicroRNA-18a prevents estrogen receptor-α expression, promoting proliferation of hepatocellular carcinoma cells. *Gastroenterology* 2009;**136**:683–93
12. Wang S-H, Yeh S-H, Lin W-H, Yeh K-H, Yuan Q, Xia N-S, Chen D-S, Chen P-J. Estrogen receptor α represses transcription of HBV genes via interaction with hepatocyte nuclear factor 4α. *Gastroenterology* 2012;**142**:989–98
13. Tang L, Pu Y, Wong DKH, Liu T, Tang H, Xiang T, Yuen MF, Ren G. The hepatitis B virus-associated estrogen receptor alpha (ER) was regulated by microRNA-130a in HepG2.2.15 human hepatocellular carcinoma cells. *Acta Biochim et Biophys Sin* 2011;**43**:640–6
14. Hamilton SR, Aaltonen LA. *World Health Organization classification of tumours. Pathology and genetics of tumours of the digestive system*. Lyon: IARC Press, 2000
15. Edmondson HA, Henderson B, Benton B. Liver-cell adenomas associated with use of oral contraceptives. *N Engl J Med* 1976;**294**:470–2
16. Lohse M, Bolger AM, Nagel A, Fernie AR, Lunn JE, Stitt M, Usadel B. RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. *Nucleic Acids Res* 2012;**40**(Web Server issue):W622–7
17. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 2009;**25**:1105–11
18. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 2009;**10**:R25
19. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 2010;**28**:511–5
20. Kalra M, Mayes J, Assefa S, Kaul A-K, Kaul R. Role of sex steroid receptors in pathobiology of hepatocellular carcinoma. *World J Gastroenterol* 2008;**14**:5945–61
21. Maynadier M, Nirde P, Ramirez JM, Cathiard AM, Platet N, Chambon M, Garcia M. Role of estrogens and their receptors in adhesion and invasiveness of breast cancer cells. *Adv Exp Med Biol* 2008;**617**:485–91
22. Teng J, Wang ZY, Jarrard DF, Bjorling DE. Roles of estrogen receptor alpha and beta in modulating urothelial cell proliferation. *Endocr Relat Cancer* 2008;**15**:351–64
23. Flanagan JM, Wilhelm-Benartzi CS, Metcalf M, Kaye SB, Brown R. Association of somatic DNA methylation variability with progression-free survival and toxicity in ovarian cancer patients. *Ann Oncol* 2013;**24**:2813–8
24. Mizoguchi Y, Takeda H, Sakagami Y, Seki S, Kobayashi K, Yamamoto S, Morisawa S. Estradiol receptors in the cytosol of peripheral blood mononuclear cells in hepatitis B virus carriers treated with interferon-alpha. *Gastroenterol Jpn* 1989;**24**:373–9
25. Mizoguchi Y, Takeda H, Kobayashi K, Yamamoto S, Morisawa S. Impairment in the response of peripheral blood mononuclear cells from asymptomatic hepatitis B virus carriers to estradiol. *Jpn J Med* 1988;**27**:183–6
26. Zhu J. DNA methylation and hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2006;**13**:265–73
27. Tischoff I, Tannapfe A. DNA methylation in hepatocellular carcinoma. *World J Gastroenterol* 2008;**14**:1741–8
28. Kos M, Reid G, Denger S, Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. *Mol Endocrinol* 2001;**15**:2057–63
29. Shuji N. Estrogen receptor 1 gene as a tumor suppressor gene in hepatocellular carcinoma detected by triple-combination array analysis. *Int J Oncol* 2013;**43**:88–94
30. Rasti M, Arabsolghar R, Khatooni Z, Mostafavi-Pour Z. p53 Binds to estrogen receptor 1 promoter in human breast cancer cells. *Pathol Oncol Res* 2012;**18**:169–75
31. Arabsolghar R, Azimi T, Rasti M. Mutant p53 binds to estrogen receptor negative promoter via DNMT1 and HDAC1 in MDA-MB-468 breast cancer cells. *Mol Biol Rep* 2012;**40**:2617–25
32. Woo HG, Wang XW, Budhu A, Kim YH, Kwon SM, Tang ZY, Sun Z, Harris CC, Thorgeirsson SS. Association of TP53 mutations with stem cell-like gene expression and survival of patients with hepatocellular carcinoma. *Gastroenterology* 2011;**140**:1063–70
33. Murphy LC, Dotzlaw H, Leygue E, Coutts A, Watson P. The pathophysiological role of estrogen receptor variants in human breast cancer. *J Steroid Biochem Mol Biol* 1998;**65**:175–80
34. Villa E, Moles A, Ferretti I, Buttafoco P, Grottola A, Del Buono M, De Santis M, Manenti F. Natural history of inoperable hepatocellular carcinoma: estrogen receptors' status in the tumor is the strongest prognostic factor for survival. *Hepatology* 2000;**32**:233–8

(Received November 6, 2013, Accepted April 2, 2014)