Hepatobiliary & Pancreatic Diseases International 18 (2019) 158–163

Contents lists available at ScienceDirect

Hepatobiliary & Pancreatic Diseases International

journal homepage: www.elsevier.com/locate/hbpd

Original Article/Liver

Alteration of oncogenic *IGF-II* gene methylation status associates with hepatocyte malignant transformation

Bo-Jun Tai[a], Min Yao[b], Wen-Jie Zheng[c], Yu-Cheng Shen[d], Li Wang[b], Jian-Ying Sun[c], Meng-Na Wu[c], Zhi-Zhen Dong[c], Deng-Fu Yao[c,*]

[a] *Department of Infectious Diseases, Affiliated Hospital of Nantong University, Nantong 226001, China*
[b] *Department of Immunology, Medical School of Nantong University, Nantong 226001, China*
[c] *Department of Oncology, Affiliated Hospital of Nantong University, Nantong 226001, China*
[d] *Department of Oncology, Affiliated Haian Hospital of Nantong University, Nantong 226601, China*

ARTICLE INFO

Article history:
Received 14 June 2018
Accepted 31 December 2018
Available online 21 January 2019

Keywords:
Hepatocellular carcinoma
Insulin-like growth factor-II
Hypomethylation
Methylation-specific PCR
Promoter
Molecular mechanism

ABSTRACT

Background: Oncogenic insulin-like growth factor-II (IGF-II) is overexpressed in hepatocellular carcinoma (HCC). The present study aimed to analyze the dynamic alteration of *IGF-II* CpG site methylation status and its molecular mechanism in HCC progression.

Methods: IGF-II alterations were observed in rat hepatocarcinogenesis models induced by 2-acetylaminofluorene. Liver IGF-II expression was compared by immunohistochemistry or tissue IGF-II specific concentration (nmol/mg protein). Status of human *IGF-II* promoter 3 (P3) or rat *IGF-II* P2 CpG site methylation was amplified by methylation-specific polymerase chain reaction (MSP). Serum IGF-II levels were quantitatively detected by an enzyme-linked immunosorbent assay.

Results: The levels of hepatic IGF-II expression were significantly elevated in the HCC group ($P < 0.001$). The unmethylation rate of *IGF-II* P3 CpG sites was 100% in the HCC-, 52.5% in the paracancerous-, and none (0%) in the distal noncancerous-tissues. Abnormal IGF-II expression was related to differentiation degree, tumor invasion, and positive HBV-DNA (all $P < 0.001$), with a negative correlation between P3 methylation degree and IGF-II expression. There was a positive correlation between liver IGF-II specific concentration and circulating IGF-II level ($r = 0.97$, $P < 0.001$). Significantly negative correlation was found between *IGF-II* P2 CpG site methylation and circulating IGF-II ($r_s = -0.89$, $P < 0.001$) or liver IGF-II level ($r_s = -0.84$, $P < 0.001$).

Conclusions: The increase of serum IGF-II and the alteration of oncogenic gene *IGF-II* methylation may be biomarkers for HCC diagnosis and DNA methylation may be the therapeutic target of HCC.

Introduction

Hepatocellular carcinoma (HCC) ranks the sixth most common cancer and the second leading cause of cancer mortality worldwide. HCC is still one of the most common tumors in China, especially in the inshore area of Yangtze River [1]. The pathogenic factors include chronic persistence hepatitis B or C virus (HBV or HCV) infection [2,3], aflatoxin B1 intake, nonalcoholic lipid accumulation and so on [4,5]. Although some new techniques have made great progress for HCC therapy, the prognosis of HCC patients is still very poor because of the late diagnosis and postoperative recurrence. Recently, a study has shown that clinical utility of new circulating tumor cells, key signal molecules, long non-coding RNA, and microRNA are the potential markers for monitoring HCC [6]. DNA cytosine methylation status, a central epigenetic modification in cellular processes [7] is closely associated with the development and progression of HCC [8]. Oncogenic insulin-like growth factor-II (IGF-II) is a fetal growth factor and a mitogenic polypeptide closely related to insulin and highly expressed in experimental liver cancer [9].

IGF-II molecular abnormalities are risk factors of HCC [10]. *IGF-II* gene contains 9 exons (E1-9) and 4 promoters (P1–P4) and has complex regulation of transcription. Different promoters initiate different mRNA transcriptions, which contribute to cell proliferation, differentiation, anti-apoptosis and invasive behaviors [11]. Hepatic and circulating IGF-II is overexpressed during HCC development. However, little is known about the relationship between *IGF-II* gene methylation and hepatocarcinogenesis [12]. In

* Corresponding author.
E-mail address: Yaodf@ahnmc.com (D.-F. Yao).

https://doi.org/10.1016/j.hbpd.2019.01.003
1499-3872/

this study, we investigated the dynamic alterations of *IGF-II* gene CpG site methylation status in different tissues of human HCC and the relationship between *IGF-II* gene and malignant transformation of hepatocytes in HCC rat model.

Methods

Human HCC tissues

Human HCC-, paracancerous- and distal noncancerous-tissues were obtained from 80 patients who underwent hepatectomy for liver cancers at the Affiliated Hospital of Nantong University, China. The livers were immediately frozen in liquid nitrogen and kept at −80 °C until use. Tissue samples were stained with Hematoxylin–Eosin (H&E) and evaluated by independent pathologists. The diagnosis of HCC was based on the criteria proposed by Chinese National Collaborative Cancer Research Group [13]. This study was approved by the Institutional Review Board of Affiliated Hospital of Nantong University. The written informed consent was obtained from the patients.

Rat HCC model

A total of 42 Sprague-Dawley rats (4–6 weeks) were provided by the Experimental Animal Center of Nantong University. Feeding conditions included clean environment, 12-h light/dark cycle, and 55% humidity according to the previously described [14]. Control group ($n=6$) were fed with normal diet, and the HCC group ($n=36$) were fed with 0.05% 2-acetylaminofluorene (2-AAF, Sigma, St Louis, USA) in diet. The rats were checked for status and sacrificed every two weeks. Rat livers were used for pathology, total protein extraction, and quantitative analysis of IGF-II. All procedures were performed according to the guidelines of the Animal Care and Use Committee of Nantong University, China.

Immunohistochemistry

Immunohistochemistry kits were purchased from the Fuzhou Maixin Biotechnology Development Co., China, and anti-human/rat IGF-II antibodies were from the Jingmei Biotechnology Development Co., Shanghai, China. Sections (5 μm) were mounted on charged glass slides, deparaffinized with xylene for 3 × 15 min and rehydrated using a graded ethanol series. Antigen retrieval was performed by placing the samples in a microwave oven for 12 min, with occasional interruption to avoid degradation by excessive heat, then treated with hydrogen peroxide, followed by incubation with primary or secondary antibodies. Streptavidin-biotin complex, amplified reagent, streptavidin-peroxidase, and substrate chromogen solutions were used (Envision system, DAKO, Glostrup, Denmark) according to the manufacturers' protocol, then counterstained with hematoxylin, rinsed with ethanol, dried and visualized by light microscopy. Anti-human/rat IGF-II antibodies were purchased from Santa Cruze (San Diego, CA, USA). The slides were read by two pathologists and the percentage of cytoplasmic staining was recorded.

DNA extraction

Total DNA were purified by the Wizard Cleanup DNA Purified kit (Promega, Madison, USA). Ten milligram of each liver was homogenized after addition of 600 μL of nuclear lysis buffer reagent for 10 s, and incubated at 65 °C for 30 min, then added 3 μL of RNase to tubes. The mixture was incubated at 37 °C for 30 min, put at room temperature for 5 min, added 200 μL of protein precipitation reagent to tubes, mixed by vortex-mixing for 20 s, and put at 0 °C for 5 min. The tubes were centrifuged at 16,000 rpm for 4 min at 4 °C. The supernatants were collected, and isopropanol (600 μL) was added and mixed gently; once obvious stripe like DNA was observed, the tubes were centrifuged at 16,000 rpm for 1 min. The supernatants were removed, the pellets washed for twice with 600 μL of 70% ethanol, mixed and centrifuged at 16,000 rpm for 1 min. The supernatants were removed again, and the DNA pellets were air dried for 15 min at room temperature and reconstituted in 100 μL of DNA Rehydration Solution and incubated at 65 °C for 1 h. The purity and concentration of DNA was measured by optical density at A260 and A280 nm in an ultraviolet spectrophotometer, and then stored at 4 °C.

Methylation-specific PCR

The modification of purified DNA was performed using the DNA Modification Kits (Qiagen Sci, MD, USA) according to manufacturer instructions. Bisulfite modification, methylation-specific PCR (MSP), and bisulfite sequencing DNA were prepared by the proteinase K method. Bisulfite treatments of *IGF-II* gene promoters were carried out as previously described [15,16]. Primers were designed using a software developed by the Johns Hopkins University (www.mspprimers.org) based on human *IGF-II* P3 (X05331) or rat *IGF-II* P2 (X17012). The sequences of methylated/unmethylated DNA primer pairs used for the MSP amplification are shown in Table 1. MSP conditions were as following: hot-start Taq polymerase (Qiagen Sci) was used with initial activation and denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 40 s; 49 °C for human *IGF-II* or 51 °C for rat *IGF-II* for 40 s; 72 °C for 40 s followed by final extension 72 °C for 10 min. Methylated DNA (M) and unmethylated DNA (U) were used as controls, respectively. The MSP products were electrophoresed on 2% agarose gels with ethidium bromide staining. The sizes were evaluated using DNA markers as molecular weight standards.

Detection of IGF-II level

Rat liver tissue (100 mg) was homogenized with extraction reagent (1:20), and phenylmethylsulfonyl fluoride was added.

Table 1
Primers of *IGF-II* P3 CpG site methylation for the MSP amplification.

Primers	Sequences (5′–3′)	Position	Size
hP3M, Forward	TTT TTA AAT TAT CGT GGT GGT TTT C	nt −889 to −768	122 bp
hP3M, Reverse	GTC TAA ATA ACT CGC CTT TAC GA		
hP3U, Forward	TTT TTA AAT TAT TGT GGT GGT TTT TG	nt −889 to −767	123 bp
hP3U, Reverse	CAT CTA AAT AAC TCA CTT TAC AAC		
rP2M, Forward	GAG AGG TTA GTT TCG GGT GTA TC	nt −1102 to −879	224 bp
rP2M, Reverse	AAA TTT ACT TAA TTA CAA ATT CGA C		
rP2U, Forward	GAG AGG TTA GTT TTG GGT GTA TTG	nt −1102 to −879	224 bp
rP2U, Reverse	AAA TTT ACT TAA TTA CAA ATT CAA C		

hP3M: human *IGF-II* gene no. 3 promoter CpG methylation; hP3U: human *IGF-II* gene no. 3 promoter CpG ummethylation; rP2M: rat *IGF-II* gene no. 2 promoter CpG methylation; rP2U: rat *IGF-II* gene no. 2 promoter CpG ummethylation; MSP: methylation- specific PCR.

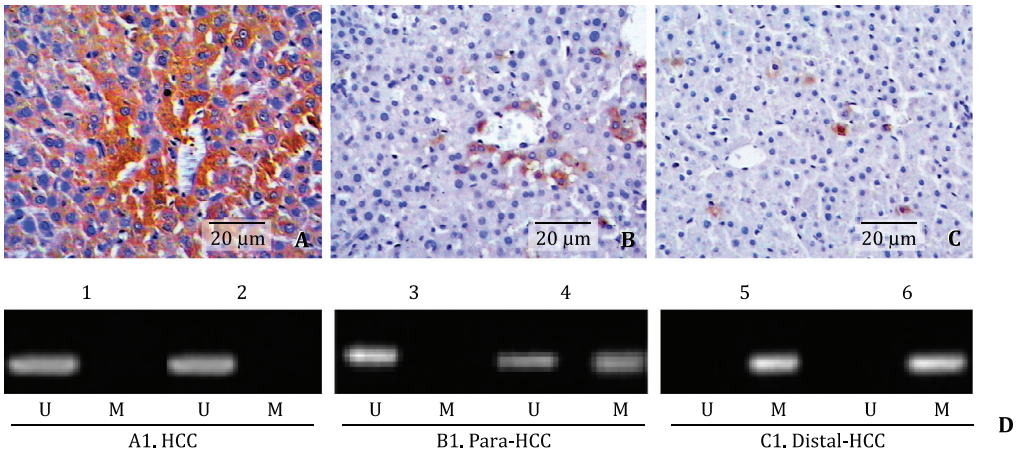

Fig. 1. IGF-II expression and methylation. **A**: HCC tissues (S-P, original magnification × 200); **B**: para-cancerous tissues (S-P, original magnification × 200); **C:** distal noncancerous tissues (S-P, original magnification × 200). **D**: *IGF-II* P3 CpG methylation. **A1**: the unmethylation fragments (123 bp) in HCC tissues; **B1**: the fragments of unmethylation (123 bp) or methylation (122 bp) in para-cancerous tissues; **C1**: the methylation (122 bp) fragments in distal cancerous tissues. HCC: hepatocellular cancinoma; Para-HCC: para-cancerous tissues; and Distal-HCC: the distal noncancerous tissues.

After shaking in ice-cold, the tubes were centrifuged according to previously described method [14]. The supernatant was transferred to a tube and the protein concentrations were detected by the Bicinchoninic Acid detection kit (Beyotime, Shanghai, China). IGF-II levels of serum or tissue supernatant were detected with ELISA (Active^{TM} IGF-II ELISA, TX, USA) according to the manufacturer's instructions. In brief, standards, controls and samples were incubated in microtitration wells, which had been coated with anti-IGF-II antibodies. After incubation and washing, the wells were treated with another anti-IGF-II detection antibody labeled with horseradish peroxidase. After a second incubation and washing, the wells were incubated with the substrate tetramethylbenzidine. An acidic stopping solution was then added and the degree of enzymatic substrate turnover was determined by dual wavelength absorbance (A) measurement at 450 nm and 620 nm.

Statistical analysis

Human livers were divided into HCC, paracancerous, and distal noncancerous groups. Rat livers according to histological examination were divided into the normal control, degeneration, precancerosis, and HCC groups. Results are expressed as mean ± standard deviation (SD). Differences among different groups were assessed by the Student's *t* test, rank sum test, Chi-square test or one-way ANOVA. Pearson's correlation (r) was applied to analyze expression between rat liver and serum IGF-II level. Spearman's correlation coefficient (r_s) for ranked data was then calculated to investigate whether there is a relationship between rat *IGF-II* gene P2 methylation and serum or liver IGF-II expression. A P value <0.05 was considered statistically significant.

Results

Patient characteristics

The patients included 64 men and 16 women, ranging from 29 to 74 years old. There were 48 cases with tumor size larger than 5 cm, 32 cases with serum AFP level more than 400 ng/mL. All cancerous-tissues were HCC; the paracancerous tissues were cirrhosis in 54 cases, chronic hepatitis in 26 cases, and atypical hyperplasia in 12 cases; and the non-cancerous tissues were cirrhosis in 32 cases, chronic hepatitis in 30 cases, and atypical hyperplasia in 18 cases.

Table 2
Incidence of *IGF-II* P3 CpG site methylation in different liver tissues.

Groups	*n*	M (%)	PM (%)	UM (%)	*Z* value[a]	*P* value[a]
HCC	80	0	0	80 (100%)	6.708	**<0.001**
Para-HCC	80	0	38 (47.5%)	42 (52.5%)	4.290	**<0.001**
Dis-HCC	80	80 (100%)	0	0 (0.0)		

[a] Compared with the distal-noncancerous group. M: methylation of liver *IGF-II* P3 CpG sites; PM: partial methylation of liver *IGF-II* P3 CpG sites; UM: unmethylation of liver *IGF-II* P3 CpG sites. HCC: the hepatocellular carcinoma group; Para-HCC: the paracancerous group; Dis-HCC: the distal-noncancerous group.

IGF-II expression and P3 CpG methylation in human HCC

The IGF-II expressions showed that IGF-II was strongly expressed in human HCC (Fig. 1A), moderately expressed in paracancerous-tissues (Fig. 1B) and weakly expressed in distal noncancerous-tissues (Fig. 1C). The methylation status of *IGF-II* P3 CpG site in different tissues was shown in Fig. 1D. The comparative analysis of methylation degree among different groups is summarized in Table 2. There was none methylational band in the HCC tissue (0%, 0/80), partial methylation in the paracancerous tissue (47.5%, 38/80), and all methylation in the distal noncancerous tissue (100%, 80/80), respectively ($P < 0.001$).

IGF-II expression and clinicopathological features of HCC

There were 69 (86.25%) cases of HCC tissues with positive IGF-II expression. The correlation between IGF-II expression and clinicopathological features of human HCC tissues are shown in Table 3. The level of IGF-II expression was significantly higher in HCC with moderate or poor differentiation than that with high differentiation (92.86% and 100 % vs 43.75%, $P < 0.001$). Hepatic IGF-II expression was markedly lower in HCC without serosa invasion than that with serosa invasion (57.69 % vs 100%, $P < 0.001$). The level of IGF-II expression in HBV DNA-positive HCC was significantly higher than that in HBV DNA-negative ones (100 % vs 54.17%, $P < 0.001$). IGF-II expression was not related to TNM staging, tumor size, tumor number, and serum AFP concentration.

Dynamic alterations of IGF-II methylation in hepatocarcinogenesis

According to the H&E staining, rat livers ($n = 42$) were divided into the control ($n = 6$), degeneration ($n = 18$), precancerosis ($n = 9$),

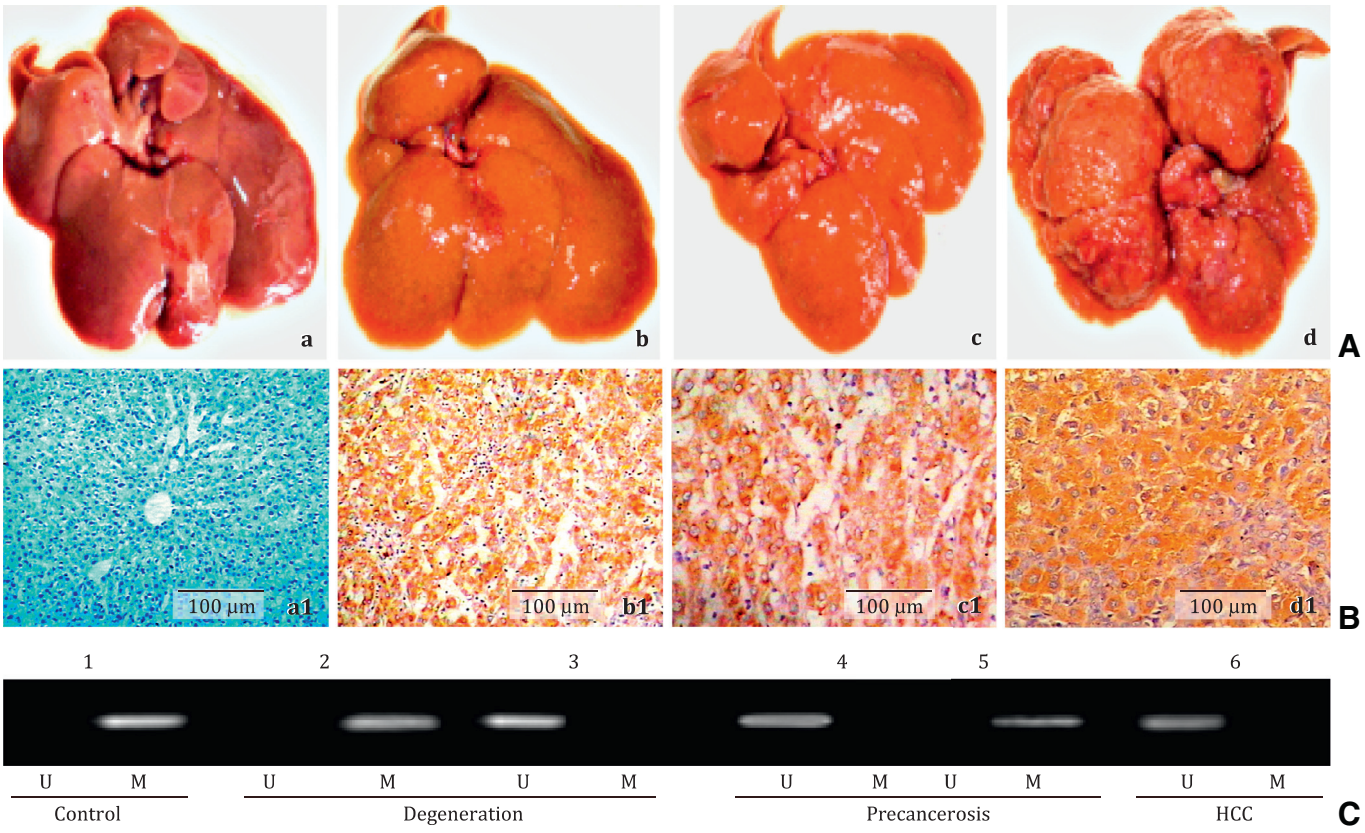

Fig. 2. Dynamic alterations of IGF-II and *IGF-II* gene P2 CpG site methylation in the process of hepatocarcinogenesis in rat. **A:** the rat liver at different stages (**a**: the control liver; **b**: the degeneration liver; **c**: the precancerous liver; and **d**: HCC). **B**: the IGF-II immunohistochemical staining ($\mathbf{a_1}$–$\mathbf{d_1}$) corresponding to above rat liver tissues (**a–d**) (S-P, original magnification × 100). **C:** the DNA methylation status (224 bp) of *IGF-II* P2 CpG site based on the accession number. M: the methylational fragments; U: the unmethylational fragments.

Table 3
Clinicopathological features of IGF-II expression in HCC tissues.

Clinicopathological features	*n*	No. IGF-II-Pos. (%)	χ^2 value	*P* value
Differentiation				
Well	16	7 (43.8%)		
Moderately	28	26 (92.9%)	13.095[a]	**<0.001**[a]
Poorly	36	36 (100%)	24.488[a]	**<0.001**[a]
TNM staging				
I–II	20	15 (75.0%)	2.846	0.092
III–IV	60	54 (90.0%)		
Serosa invasion				
With	54	54 (100%)	26.488	**<0.001**
Without	26	15 (57.7%)		
Tumor size (cm)				
<5	32	26 (81.3%)	1.124	0.289
≥5	48	43 (89.6%)		
AFP (ng/mL)				
<400	48	44 (91.7%)	1.022	0.312
≥400	32	27 (84.4%)		
Tumor number				
Single	31	26 (83.9%)	0.242	0.623
Multiple	49	43 (87.8%)		
HBV DNA				
Positive	56	56 (100%)	29.758	**<0.001**
Negative	24	13 (54.2%)		

[a] Compared with the well differentiated group. AFP: α-fetoprotein; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; No. IGF-II-Pos.: the number of positive IGF-II expression in HCC tissues; TNM: tumor node metastasis.

Table 4
Incidence of *IGF-II* gene P2 CpG site methylation in rat HCC.

Groups	*n*	M	PM	UM	MR (%)	*Z* value[a]	*P* value[a]
Normal control	6	6	0	0	6 (100%)		
Degeneration	18	13	2	3	15 (83.3%)	1.411	0.158
Precancerosis	9	0	2	7	2 (22.2%)	3.586	**<0.001**
HCC	9	0	0	9	0	3.742	**<0.001**

[a] Compared with the normal control group. M: methylation of rat liver *IGF-II* P2 CpG sites; PM: partial methylation of rat liver *IGF-II* P2 CpG sites; UM: unmethylation of rat liver *IGF-II* P2 sites; MR: the methylational rate of rat liver *IGF-II* P2 CpG sites.

and HCC ($n = 9$) groups. The relationship between IGF-II expression and *IGF-II* gene P2 CpG methylation was explored in rat hepatocarcinogenesis model (Fig. 2). Immunohistochemistry showed that the expression of IGF-II was gradually increased following the hepatocarcinogenesis. The *IGF-II* gene P2 CpG site methylation status showed that DNA fragments were all methylated in the control group, partially methylated in the degeneration or precancerous groups, and all unmethylated in the HCC group. The comparative analysis of *IGF-II* gene methylation degree in different groups was summarized in Table 4. Significantly low methylation rate (22.2% and 0%, respectively) was observed in the precancerous and HCC groups, and high methylation rate (100% and 83.3%, respectively) in the normal control and degeneration groups.

IGF-II expression with malignant transformation of hepatocytes

The expressions of circulating or liver IGF-II with the malignant transformation of rat hepatocytes are shown in Table 5. The quantitative analysis showed that blood and liver IGF-II were significantly increased following the malignant transformation of hepatocytes and the blood and liver IGF-II level were positively correlated ($r = 0.97$, $P < 0.001$). Also, there was significantly

Table 5
Expression of IGF-II in serum and liver of HCC rat.

Groups	*n*	Serum IGF-II (nmol/L)			Liver IGF-II (nmol/mg protein)		
		Mean ± SD	*q* value[a]	*P* value[a]	Mean ± SD	*q* value[a]	*P* value[a]
Normal control	6	199.6 ± 25.4			69.7 ± 6.0		
Degeneration	18	232.2 ± 57.9	1.18	0.643	73.2 ± 17.0	1.63	0.723
Precancerosis	9	365.5 ± 32.1	5.36	**0.040**	93.7 ± 11.2	4.18	**0.036**
HCC	9	600.4 ± 150.1	12.97	**<0.001**	107.6 ± 9.8	6.75	**0.004**

[a] Compared with the normal control group. Liver IGF-II: the IGF-II specific concentration (nmol/per mg protein) in rat livers.

negative correlation between rat *IGF-II* gene P2 CpG site methylation and circulating IGF-II level ($r_s = -0.89$, $P < 0.001$) or liver IGF-II concentration ($r_s = -0.84$, $P < 0.001$).

Discussion

Hepatocarcinogenesis is a multi-factor, multi-step, and complex process. Genetic and epigenetic changes regulate the expression of cancer-related genes, including DNA methylation, histone modification, and chromosome remodeling [6,11]. The mechanisms of DNA methylation within CpG islands in HCC include: oncogenes activated by DNA methylation (c-Jun, c-Myc and c-Ha-ras) and tumor suppressor genes inactivated (P16) that have negative relationship with genes expression. IGF-II is speculated to serve as an autocrine growth factor in various cancers, and most of the cirrhotic and HCC tissues express IGF-II [17]. In this study, the dynamic changes of *IGF-II* gene promoter methylation status were investigated in tissues of human HCC or rat HCC.

Many studies showed that the gene methylation status was closely related to the development of HCC [16–18]. IGF-II is highly expressed in the fetal liver and new born, which is mainly based on activation of P2–P4. IGF-II expression is strongly reduced in adulthood, mainly based on activation of P1 [7,11]. IGF-II expression was elevated in rat preneoplastic lesions and in HCC tissues. The frequencies of *IGF-II* gene P3 methylation were lost in HCC tissues, half of methylation or unmethylation in paracancerous tissues, and all methylation in distal noncancerous tissues, suggesting the correlation between loss of *IGF-II* gene methylation and HCC progression, and it could be an early event in HCC formation.

Abnormal IGF-II expressions in HCC tissues are significantly related to the loss of *IGF-II* gene methylation, especially in patients with HBV or HCV infection [19,20]. The expression of IGF-II in HCC tissues is associated with degree of differentiation, serosa invasion, and HBV infection. Alteration of *IGF-II* gene methylation should be a central epigenetic modification that has essential roles in cellular processes including genome regulation, development and disease. The methylation rates of *IGF-II* gene P3 in HBV-related HCC patients were significantly decreased, suggesting the negative correlation between methylation status of *IGF-II* gene P3 and HCC, and the epigenetic analysis of *IGF-II* gene or other HCC related genes could be useful to understand carcinogenic processes and to develop a novel targeted therapy for HCC.

In this study, the dynamic alterations of liver IGF-II, circulating IGF-II and *IGF-II* gene methylation were identified with rat model of hepatocarcinogenesis [21]. The quantitative analysis of IGF-II in either blood or liver demonstrated that its expression was positively related with malignant transformation of rat liver cells. There was a closely positive correlation between liver IGF-II specific concentration and serum IGF-II level. For the first time, significantly negative correlation was found between rat *IGF-II* gene CpG site methylation and serum or liver IGF-II expression. This alteration was in agreement with the presence of an increasing methylation gradient from the cancer to the distal, with a higher level of methylation from well to poorly differentiated HCC, suggesting that the oncogenic *IGF-II* activation by regulating gene methylation would promote malignant transformation of hepatocytes.

In conclusion, the present study firstly reported that elevated expression of IGF-II during HCC formation was the result of *IGF-II* gene dynamic demethylation. Serum IGF-II may be a biomarker for HCC diagnosis and DNA methylation may be the therapeutic target of HCC.

Contributors

TBJ and YM contributed equally to this work, designed and wrote the article. SYC, SJY, and WMN performed the study. ZWJ and WL analyzed the data. DZZ and YDF are the guarantors.

Funding

This study was supported partly by grants from the National Natural Science Foundation (81673241, 81702419, 81872738, 81873915), the Jiangsu Medical Science (BE2016698) & Graduate Innovation (KYCX17_1934), and the International S. & T. Cooperation Program (2013DFA32150) of China.

Ethical approval

This study was approved by the Institutional Review Board of Affiliated Hospital of Nantong University. The written informed consent was obtained from the patients.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

[1] Chen JG, Zhu J, Zhang YH, Zhang YX, Yao DF, Chen YS, et al. Cancer survival in Qidong between 1972 and 2011: a population-based analysis. Mol Clin Oncol 2017;6:944–954.
[2] Tong HV, Bock CT, Velavan TP. Genetic insights on host and hepatitis B virus in liver diseases. Mutat Res Rev Mutat Res 2014;762:65–75.
[3] Matsuura K, Tanaka Y. Host genetic variations associated with disease progression in chronic hepatitis C virus infection. Hepatol Res 2018;48:127–133.
[4] Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. CA Cancer J Clin 2012;62:394–399.
[5] Ubagai T, Kikuchi T, Fukusato T, Ono Y. Aflatoxin B1 modulates the insulin-like growth factor-2 dependent signaling axis. Toxicol In Vitro 2010;24:783–789.
[6] Jain S, Singhal S, Lee P, Xu R. Molecular genetics of hepatocellular neoplasia. Am J Trans Res 2010;2:105–118.
[7] Breuhahn K, Schirmacher P. Reactivation of the insulin-like growth factor-II signaling pathway in human hepatocellular carcinoma. World J Gastroenterol 2008;14:1690–1698.
[8] Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286–298.
[9] Mukherjee B, Ghosh S, Das T, Doloi M. Characterization of insulin-like-growth factor II (IGF II) mRNA positive hepatic altered foci and IGF II expression in hepatocellular carcinoma during diethylnitrosamine-induced hepatocarcinogenesis in rats. J Carcinog 2005;4:12.

[10] Tang SH, Yang DH, Huang W, Zhou HK, Lu XH, Ye G. Hypomethylated P4 promoter induces expression of the insulin-like growth factor-II gene in hepatocellular carcinoma in a Chinese population. Clin Cancer Res 2006;12:4171–4177.
[11] Scharf JG, Braulke T. The role of the IGF axis in hepatocarcinogenesis. Horm Metab Res 2003;35:685–693.
[12] Qian J, Yao D, Dong Z, Wu W, Qiu L, Yao N, et al. Characteristics of hepatic IGF-II expression and monitored levels of circulating IGF-II mRNA in metastasis of hepatocellular carcinoma. Am J Clin Pathol 2010;134:799–806.
[13] Ministry of Health of the People's Republic of China Updated standards for the diagnosis and treatment of primary liver cancer. Zhonghua Gan Zang Bing Za Zhi 2012;20:419–426.
[14] Qiu LW, Yao DF, Zong L, Lu YY, Huang H, Wu W, et al. Abnormal expression of insulin-like growth factor-II and its dynamic quantitative analysis at different stages of hepatocellular carcinoma development. Hepatobiliary Pancreat Dis Int 2008;7:406–411.
[15] Huang Z, Bassil CF, Murphy SK. Methylation-specific PCR. Methods Mol Biol 2013;1049:75–82.
[16] Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93:9821–9826.
[17] Villanueva A, Portela A, Sayols S, Battiston C, Hoshida Y, Méndez-González J, et al. DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma. Hepatology 2015;61:1945–1956.
[18] Jain S, Chang TT, Hamilton JP, Lin SY, Lin YJ, Evans AA, et al. Methylation of the CpG sites only on the sense strand of the APC gene is specific for hepatocellular carcinoma. PLoS One 2011;6:e26799.
[19] Couvert P, Carrié A, Tezenas du Montcel S, Vaysse J, Sutton A, Barget N, et al. Insulin-like growth factor 2 gene methylation in peripheral blood mononuclear cells of patients with hepatitis C related cirrhosis or hepatocellular carcinoma. Clin Res Hepatol Gastroenterol 2012;36:345–351.
[20] Liu XY, Tang SH, Wu SL, Luo YH, Cao MR, Zhou HK, et al. Epigenetic modulation of insulin-like growth factor-II overexpression by hepatitis B virus X protein in hepatocellular carcinoma. Am J Cancer Res 2015;5:956–978.
[21] Martinez-Quetglas I, Pinyol R, Dauch D, Torrecilla S, Tovar V, Moeini A, et al. IGF2 is up-regulated by epigenetic mechanisms in hepatocellular carcinomas and is an actionable oncogene product in experimental models. Gastroenterology 2016;151:1192–1205.