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Methylation mediated Gadd45 β enhanced the chemosensitivity of hepatocellular carcinoma by inhibiting the stemness of liver cancer cells

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Abstract

Background: Defects of the growth arrest DNA damage-inducible gene 45 β (Gadd45 β) play an important role in the progression of tumor and confer resistance to chemotherapy. However, the role of Gadd45 β in the apoptosis of hepatocellular carcinoma is still not clear. Purpose of this study was to explore the effect of Gadd45 β on the apoptosis of liver cancer cells, and the possible mechanism was examined.

Result: In this study, we first confirmed the decreased expression of Gadd45 β in human liver cancer tissues and human liver cancer cell lines, when compared to the peri-tumor liver tissue and normal liver cells. And, it was found that Gadd45 β could inhibit the stemness of liver cancer cells, enhancing the apoptosis of cancer cells induced by chemotherapy. Furthermore, the results showed that HCC tissues and cell lines showed a higher methylation status in Gadd45 β promoter than that in peri-tumor tissues and normal liver cells. Methylation was then reversed by pretreatment of SMMC-7721 and Hep-3B with 5-azacytidine which is the DNA methyltransferase inhibitor. And the 5-azacytidine decreased the stemness of SMMC-7721 and Hep-3B, enhanced the sensitivity of SMMC-7721 and Hep-3B to cisplatin.

Conclusions: Methylation mediated Gadd45 β expression inhibited the stemness of liver cancer cells, promoting the chemotherapy-induced apoptosis. Thus Gadd45 β may be the potential target for enhancing the chemosensitivity of human hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, Gadd45 β methylation, Apoptosis, Stemness

Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths. There are approximately 750,000 new cases of liver cancer each year worldwide [1]. Since the symptom of early-stage HCC is not obvious, hepatocellular carcinoma is often diagnosed at intermediate or advanced stages, when only chemoembolization and sorafenib have shown benefits [2]. However,

HCC has a very poor survival rate and prognosis. Thus, it is necessary to find an effective chemotherapy target for the treatment of HCC.

The Growth Arrest and DNA damage-inducible 45 (Gadd45) gene family contains three members, Gadd45 α , Gadd45 β , and Gadd45 γ . Gadd45 protein are mainly located within the cell nucleus. They are a group of stress-induced protein family, a variety of stresses stimulation can induce their expression, such as UV-radiation, ionizing irradiation, medium starvation, and alkylating agent [3]. The Gadd45 proteins play important roles in the multiple cellular processes, including cell cycle control, proapoptosis, DNA repair [4–7]. These are

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important event for cellular defense against DNA damage and tumorigenesis. Therefore, the deficiency of Gadd45 gene may contribute to the tumorigenesis. It was found that Gadd45 α -deficient mice increased cancer susceptibility [8] [9], and it was also observed that Gadd45 was frequently deficient in tumor cells [10, 11]. In addition, the study indicated that the induction of Gadd45 β promoted the therapeutic drug induced apoptosis in hepatocellular carcinoma [12]. However, the mechanism is still not clear.

The role of Gadd45 in apoptosis is complicated in tumor cells. Gadd45 proteins were originally reported as a pro-apoptotic protein; but recent studies showed that Gadd45 α and Gadd45 β increased tumor cell survival under treatment with chemotherapeutic drugs [13]. Gadd45 β proteins are frequently particularly decreased expression in hepatocellular carcinoma (HCC) [10, 14, 15]. However, the role of Gadd45 β in the apoptosis of hepatocellular carcinoma is still not clear. In current study, we found that the overexpression of Gadd45 β enhanced the chemotherapy induced apoptosis in hepatocellular carcinoma. And the mechanism may depend on the inhibition of stemness of liver cancer cells induced by induction of Gadd45 β .

Methods

Cell culture, transfection, and drug treatment

Human HCC cell line SMMC-7721 and Hep-3B, and normal liver cell line L-02 were acquired from the Chinese Academy of Sciences Cell Bank. L-02 cells were cultured in 1640 medium, SMMC-7721 and Hep-3B cells were cultured in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 unites/ml of penicillin, and 100 μ g/ml of streptomycin (all from Invitrogen) in a humidified atmosphere of 5% CO₂ at 37 °C.

For overexpression of Gadd45 β , Gadd45 β -expressing adenovirus were transfected into SMMC-7721 and Hep3B cells according to the manufacturer's instructions. Briefly, both cells were seeded (2×10^5 cells/well) into 12-well plates overnight. Gadd45 β expressing lentivirus were then transfected into the cells. After 24 h of transfections, fresh medium was changed to remove virus. Seventy-two hours after the transfection, the effect of viral on cell were analyzed. Two weeks after transfection, Hep3B^{+Gadd45 β} and SMMC-7721^{+Gadd45 β} cells were collected by flow cytometry sorting.

5-Azacytidine was purchased from Sigma-Aldrich. SMMC-7721 and Hep-3B cells were pretreated with 10 μ m of 5-Azacytidine for 72 h. For determining the sensitivity to cisplatin, after pretreatment of 5-Azacytidine for 72 h, SMMC-7721 and Hep-3B cells were seeded for 24 h and treated with cisplatin (2 μ g/ml).

Patients and specimens

100 cases of HCC and peri-tumor tissues were acquired from Shanghai Eastern Hepatobiliary Surgery Hospital. Informed consent was obtained from each patient under a protocol approved by the Hospital Research Ethics Committees. The patients' demographics and baseline characteristics are summarized in Table 1. Tumor cell differentiation was assessed basing on the H&E staining as previous describe [10]. Briefly, well differentiated HCC was characterized with minimal atypia and an increased nuclear/cytoplasmic ratio; moderately differentiated HCC were arranged in trabeculae of more cells in thickness and possessed abundant eosinophilic cytoplasm and round nuclei; poorly differentiated HCC had an increased nuclear/cytoplasmic ratio and frequent pleomorphism and bizarre giant cells.

Immunohistochemistry

Tumor sections were cut and fixed in 4% formaldehyde solution. Endogenous peroxidase activity was blocked by methanol-3% H₂O₂. The primary rabbit anti-human polyclonal Gadd45 β antibody (dilution 1:100; Abcam, Cambridge, UK) was used to incubate the tissue sections in a humid container at 4 °C for overnight, goat anti-rabbit antibody (dilution 1:200; Invitrogen, Carlsbad, CA, USA) was used as secondary antibody, and the sections were washed with PBS. Granular cytoplasmic stain with yellow color was considered as positive, no staining was considered as negative when observed under a Leica DMRA microscope (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK). Furthermore, the positive staining of Gadd45 β was classified: + represents less than 30%

Table 1 Relationship between patient demographic features, HCC differentiation and Gadd45 β expression (n = 100)

Feature	n = 100	No. expression		P
		Gadd45 β (0)	Gadd45 β (+ to +++)	
Age (years)				0.18
< 65	28	20	8	
\geq 65	72	60	12	
Gender				0.66
Male	65	40	25	
Female	35	20	15	
Hepatitis B				0.58
Positive	70	55	15	
Negative	30	25	5	
Differentiation				0.009
Well	21	10	11	
Moderate	17	12	5	
Poor	62	58	4	

P < 0.05 was considered to be statistically significant

staining; ++ represents 30–70% staining; +++ represents more than 70% staining.

Real-time PCR and reverse transcription PCR

To further validate the results of IHC study, Real-Time PCR were performed to examine the Gadd45 β expression in fresh liver cancer tissue and surrounding non-neoplastic liver tissue. The selected tissues were first performed the RNA isolation by DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The cDNA was reverse-transcribed from total RNA using bestar qPCR RT kit (DBI[®] Bioscience, Germany). The real-time RT-PCR was then carried out by using the SYBR Green Master Mix Kit (DBI[®] Bioscience, Germany). To examine the Gadd45 β expression in liver cancer cells and normal liver cells. The reverse transcription PCR was performed by using 2 \times Taq PCR Master Mix (DBI[®] Bioscience, Germany). In order to normalize the Gadd45 β mRNA expression, the endogenous GAPDH mRNA was used. Primers for GAPDH determination were: 5'-AC AACTTTGGTATCGTGGAAGG-3'(forward), 5'-GCCATCACGCCACAGTTTC-3'(reverse); Primers for Gadd45 β determination were: 5'-ACGAGTCGGCCAAGTTGATG-3'(forward), 5'-GGATGAGCGTGAAGTGGATTT-3'(reverse). In addition, to examine the stem character of cells, the following primers were used. Primers for SOX9 determination were: 5'-AGCGAACGCACATCAAGAC-3'(forward), 5'-CTGTAGGCGATCTGTTGGGG-3'(reverse); Primers for OCT4 determination were: 5'-CTGGTTGATCCTCGGACCT-3'(forward), 5'-CCATCGGAGTTGCTCTCCA-3'(reverse). The PCR conditions were set as following: an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s, 72 °C for 1 min.

Western-blot analysis

The expression levels of Gadd45 β protein in cells and tissues were assessed by western blot assay. In detail, the cell and tissue samples were firstly collected, and then lysed. The protein concentration of the whole lysates was quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The following antibodies were used in the experiment: rabbit anti-human polyclonal antibody to Gadd45 β (dilution 1:2000; Abcam, Cambridge, UK), rabbit anti-GAPDH antibody (dilution 1:1000; Abcam); the secondary goat anti-rabbit antibody (dilution 1:5000; Abcam). In addition, to examine the stem character of cells, the used antibodies were specific for SOX9, OCT4, GAPDH (Abcam), and goat anti-rabbit secondary antibody (Abcam).

Colony formation assay

The transfected and non-transfected SMMC-7721, Hep3B (1×10^3) were seeded in plates. The cells were

incubated for 2 weeks to allow the development of colonies. Cell culture medium were moved, and the exposed cells were fixed in ethanol and stained with 2% crystal violet. Then the colony formation of transfected and non-transfected cells can be observed and counted.

Methylation analysis

To determine whether the decreased expression of Gadd45 β is associated with hypermethylation in promoter, methylation-specific PCR (MSP) assay was used to measure the hypermethylation status of Gadd45 β promoter as previously described [16]. Before MSP assay, the DNA samples were pre-treated, genomic DNA was isolated by DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and treated with bisulfate modification using DNA modification kit (QIAGEN, Hilden, Germany). Then bisulfate-treated DNA was used to the MSP amplification using the methylated and unmethylated Gadd45 β primers as previous study [17]. The primers for amplification of methylated DNA were 5'-TTCGAAAGTTCGGGTCGTTTCGCGC-3'(forward) and 5'-GGGACCGAATAAATAACCGCG-3'(reverse); the primer for amplification of unmethylated DNA were: 5'-AAAGTTTGGGTTGTTTTGTGT-3'(forward) and 5'-ACCAAATAAATAACCACA-3'(reverse). PCR products were 137 and 128-bp in the methylated specific and unmethylated primers system, respectively.

Flow cytometric analysis

The cell apoptosis was measured by an Annexin-APC assay via flow cytometry according to the manufacturer's instructions (Nanjing Keygen Biotech, China, Cat. KGA108). Briefly, SMMC-7721, Hep3B cells treated with 5-AZA and nontreatment were seeded in 6-well plates to 70–80% cell confluence. The cells were treated with cisplatin for 10 h. Then the cells were collected by trypsinization, washed twice with phosphate-buffered saline (PBS) and resuspended in 200 μ l of binding buffer containing 2 μ l Annexin and 2 μ l PI, incubated for 15 min at room temperature in the dark. After the incubation, at least 10^5 cells were measured with flow cytometry.

Statistical analysis

Statistical analysis of data was performed by Student's *t* test. Data are presented as mean \pm SD. Differences between categorical variables were assessed by the Chi square test. *P* < 0.05 was considered to be statistically significant.

Results

Down-regulation of Gadd45β expression in HCC and patient demographic features

We firstly examined Gadd45β expression in one hundred of HCC cases and peri-tumor tissues by immunohistochemistry (IHC) staining. Figure 1a showed the typical image of negative and positive staining of Gadd45β in tumor and peri-tumor tissues, respectively. Furthermore, according to the staining pattern of Gadd45β as described above, we found that the peri-tumor tissue showed 80% (80 of 100) positive staining, while tumor tissue showed only 20% (20 of 100) positive staining (Fig. 1b). To validate the IHC analyses, 20 paired of fresh HCC and peri-tumor samples were further examined by the RT-PCR study. The quantitative analysis showed that Gadd45β mRNA level in liver cancer tissue were readily lower than that in peri-tumor tissues (**P < 0.01; ***P < 0.001) (Fig. 1c). Furtherly, western blot results also showed the down-regulation of Gadd45β expression in tumor tissues, when compared to the peri-tumor tissues (Fig. 1d).

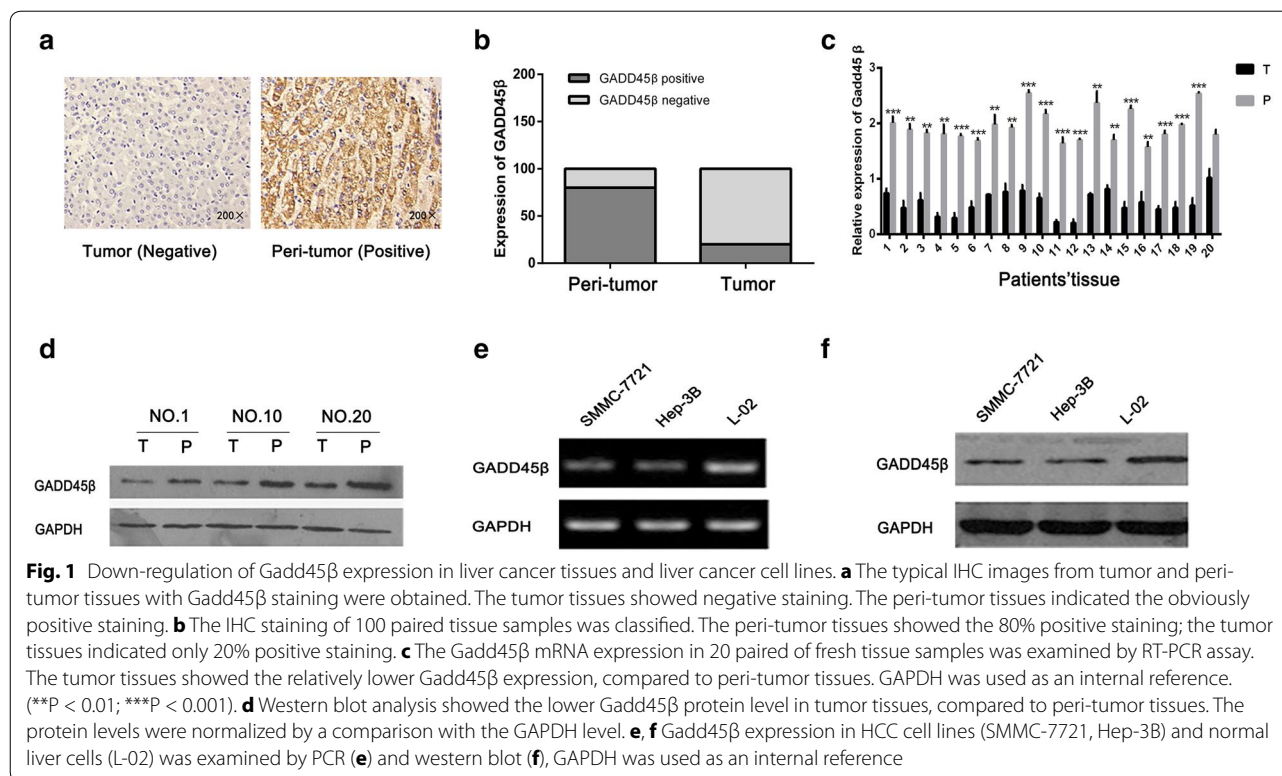
To further confirm the difference of Gadd45β expression in tumor and peri-tumor, two different HCC cell lines (SMMC-7721, Hep-3B) and normal liver cell (L-02) were selected to examine the Gadd45β mRNA expression by PCR assay. As shown in Fig. 1e, Gadd45β mRNA level in all HCC cell lines was relatively lower than that in normal liver cell line. Similarity, western blot assay also

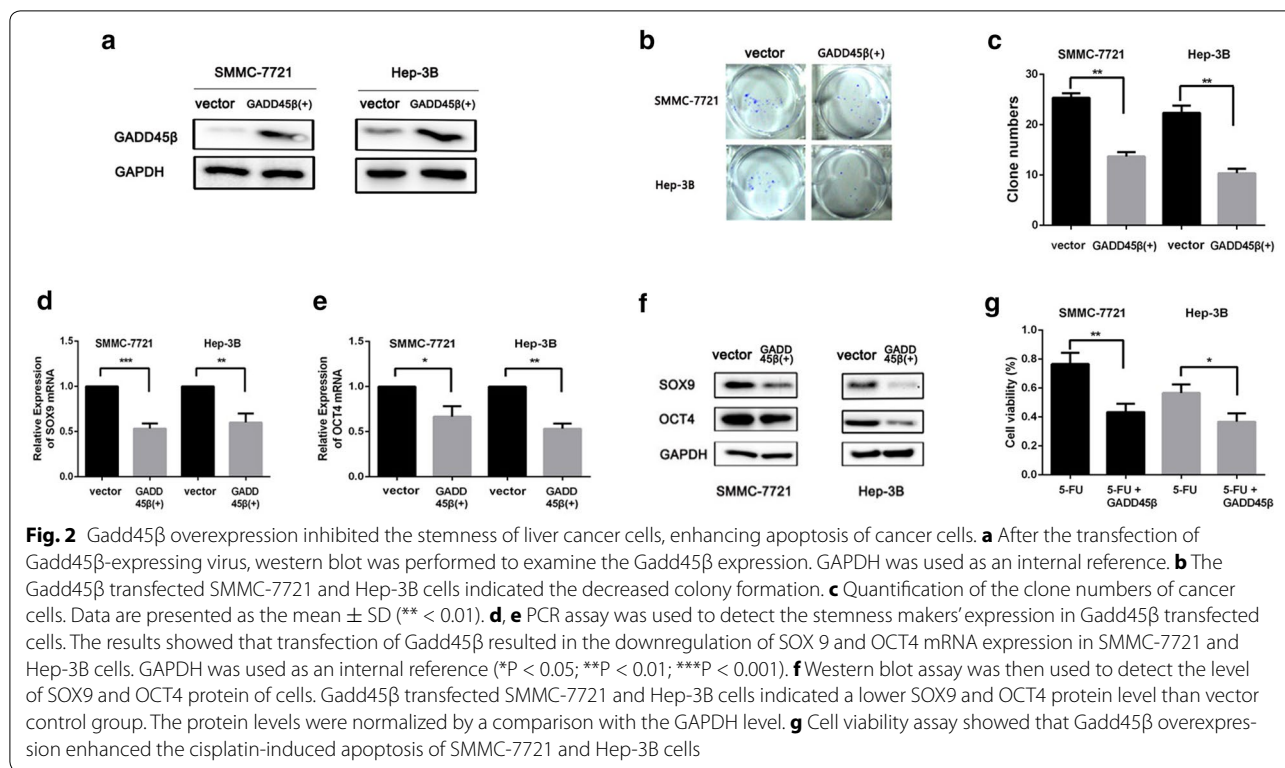
showed that expression of Gadd45β protein was lower in HCC cell lines, when compared to normal liver cell line (Fig. 1f). From these results, we confirmed that expression of Gadd45β was significantly decreased in human liver tumor cells and tissues, when compared to normal liver cells and peri-tumor tissues.

Table 1 showed the 100 cases of patients' demographics, clinicopathological factors, and the status of Gadd45β expression. Chi square test was used to examine the association of patients age, gender, hepatitis B, and Gadd45β expression, the result showed that there are no significant difference; However, there was a correlation between Gadd45β expression and HCC differentiation, this association was tested by Chi square (P < 0.05). The well differentiated HCC samples showed the higher expression of Gadd45β. The higher deficiencies of Gadd45β expression was observed in poorly differentiated samples. This suggested us that Gadd45β may play an important role in the stemness of liver cancer cells.

Gadd45β expression inhibited the stemness properties of liver cancer cells

To further determine the impact of Gadd45β on liver cancer cells, Gadd45β-expressing adenovirus were firstly transfected into SMMC-7721 and Hep3B cells. Western blot assay was performed to determine the Gadd45β expression (Fig. 2a). Then, the ability of colony formation



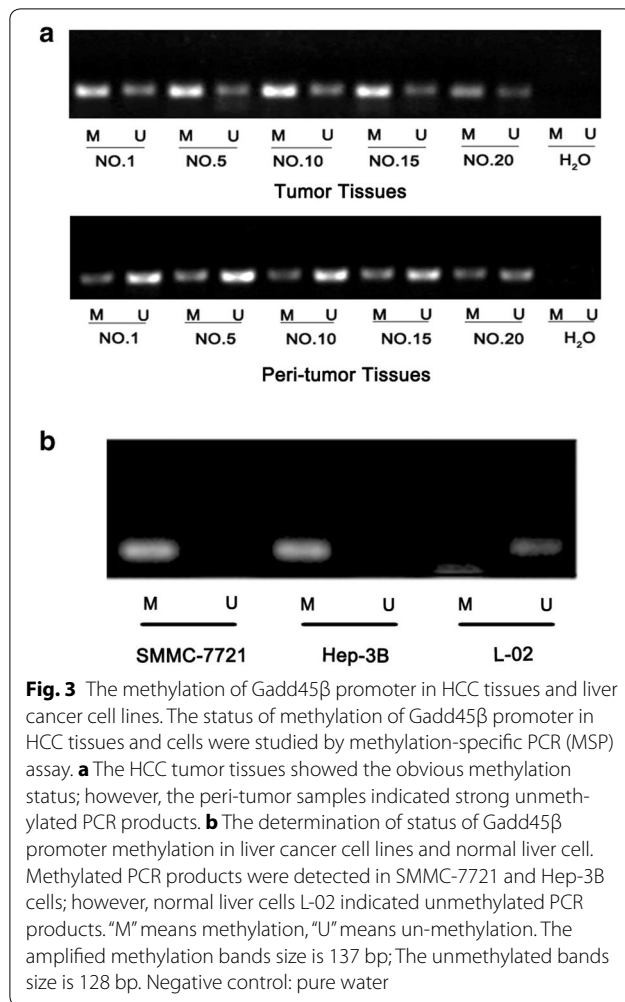


in the transfected cells was examined. It was found that Gadd45β transfected SMMC-7721 and Hep-3B cells showed noticeable decrease in the colony formation, when compared with control group (**P < 0.01) (Fig. 2b, c), suggesting that the stemness of SMMC-7721 and Hep-3B cells may be inhibited by the overexpression of Gadd45β. Then, the expression of stem cell surface markers (SOX9 and OCT4) was assessed in Gadd45β transfected SMMC-7721 and Hep-3B cells, the results showed that SOX9 and OCT4 expression were reduced at both mRNA and protein levels in Gadd45β transfected liver cancer cells (*P < 0.05; **P < 0.01; ***P < 0.001) (Fig. 2d, e, f). Finally, to assess the effect of Gadd45β proteins on the apoptosis of liver cancer cells, cell viability assay was further used to assess the degree of apoptosis of cells with different treatment. The results showed that Gadd45β promoted the cisplatin induced apoptosis of SMMC-7721 and Hep-3B (*P < 0.05; **P < 0.01) (Fig. 2g). Therefore, we concluded that Gadd45β overexpression inhibited the stem cell properties of liver cancer cells, enhancing the chemosensitivity.

The methylation of Gadd45β promoter in HCC tissues and liver cancer cell lines

It has been reported that methylation of promoter can silence CpG islands, and which was thought to be a epigenetic mechanism to inactive gene functions

[18]. Furthermore, the study had shown that promoter methylation mediated the repression of Gadd45α and Gadd45β in gastric cardia adenocarcinoma [19]. We suspected that the silence of Gadd45β may be associated with the methylation of promoter in HCC. Thereby, the status of hypermethylation of CpG island was detected in HCC samples. The MSP primers were designed according to previous report [19]; the methylation-specific PCR (MSP) assay was performed in the selected clinical tumor specimens and surrounding peri-tumor tissues to qualitatively observe the methylated status of Gadd45β promoter. Among these five patients' tumor samples, all PCR products could be amplified by the methylation-specific and unmethylated primers, but strong PCR products could be observed in the MSP system where the methylated primers were used. However, in peri-tumor samples, unmethylated primers were obviously amplified (Fig. 3a). Similarity, the normal liver cell L-02 showed an apparent un-methylation PCR products. Meanwhile, Gadd45β non-expressing HCC cell lines indicated the obvious methylation PCR products (Fig. 3b). The result suggested that there exists the obviously unmethylated Gadd45β in the liver peri-tumor tissues and normal cell, and where Gadd45β proteins were higher expressed; however, compared with peri-tumor tissues and normal liver cells, Gadd45β promoter were mainly methylated in tumor tissues and cancer cells, and where Gadd45β



expression were mainly repressed. These results suggest that Gadd45 β promoter methylation may influence the protein expression of Gadd45 gene in tumor tissues.

Up-regulation of Gadd45 β by pretreatment with 5-azacytidine increased sensitivity of liver cancer cells to cisplatin, and decreased the stemness of HCC cell lines

5-azacytidine (5-AZA) is the DNA methyltransferase (DNMT) inhibitor, thus we hypothesized that induction of Gadd45 β by pretreatment of 5-azacytidine would increase sensitivity of cancer cells to chemotherapy. Firstly, the depletion of Gadd45 β methylation by 5-AZA was examined by MSP assay. The result showed that 5-AZA inhibited efficiently the Gadd45 β methylation of SMMC-7721 and Hep-3B cells (Fig. 4a). And western blot confirmed that Gadd45 β expression was unregulated in cells with treatment of 5-AZA (Fig. 4b). Furthermore, RT PCR assays showed that the 5-azacytidine also inhibited the SOX9 and OCT4 mRNA expression of HCC cell lines (Fig. 4c, d). Then the cells with or without the

pretreatment of 5-AZA were treated with Cisplatin (2 μ g/ml), flow cytometry showed that the increased apoptosis was observed in SMMC-7721 and Hep-3B cells with the pretreatment of 5-azacytidine, when compared to the cells without the pre-treatment of 5-azacytidine (Fig. 4e). And quantitative analysis of apoptosis confirmed that these differences were significant (**P < 0.01) (Fig. 4f, g). Thereby, these results confirmed the combination of 5-azacytidine and cisplatin could promote the chemotherapy-mediated apoptosis in SMMC-7721 and Hep-3B cells by inhibiting the stemness of the liver cancer cells.

Discussion

The tumor cellular response to chemotherapy still remain complex. Gadd45 proteins could rapidly response to genotoxic stress. However, the exact function of Gadd45 proteins in the response of tumor cells to genotoxic stress is still controversial. It was found that Gadd45 α and Gadd45 β proteins play a dual role in the apoptosis of tumor cells induced by genotoxic stress, showing both apoptotic and antiapoptotic function [20, 21]. In this study, the role of Gadd45 β in the apoptosis of liver cancer cells was studied.

Firstly, the expression of Gadd45 β in one hundred cases of HCC and surrounding peri-tumor tissues were studied. IHC results showed that the cancerous tissue displayed lower Gadd45 β level than that in precancerous tissues. The results were further validated by PCR and western blot study. Furthermore, the tested liver cancer cell lines also showed a lower expression of Gadd45 β , when compared to normal liver cell. And there exist an association between Gadd45 β and HCC differentiation. Our observation keep consistent with many other studies [10, 22]. In order to study the impact of Gadd45 β on liver cancer cells, Gadd45 β -expressing adenovirus were transfected into SMMC-7721 and Hep-3B cells, we found that Gadd45 β overexpression inhibited the stemness properties of cells, including the downregulation of SOX9 and OCT4 expression, reduction of colony formation. The stemness characters of cancer cells plays a critical role in the chemoresistance of cancer. Thus, we next examined the impact of Gadd45 β on the chemotherapy-induced apoptosis in SMMC-7721 and Hep-3B cells, it was found that Gadd45 β overexpression enhanced the cisplatin-induced apoptosis in SMMC-7721 and Hep-3B cells. Furthermore, it was found that methylation was involved in the downregulation of Gadd45 β , and induction of Gadd45 β by treatment of cells with DNA methyltransferase inhibitor (5-azacytidine) also increased the sensitivity of cancer cells to chemotherapy. Thus, Gadd45 β may be a potential target for the therapy of hepatocellular carcinoma.

The exact mechanism of Gadd45 protein down-expression in cancer is still not clear. Up to now, the

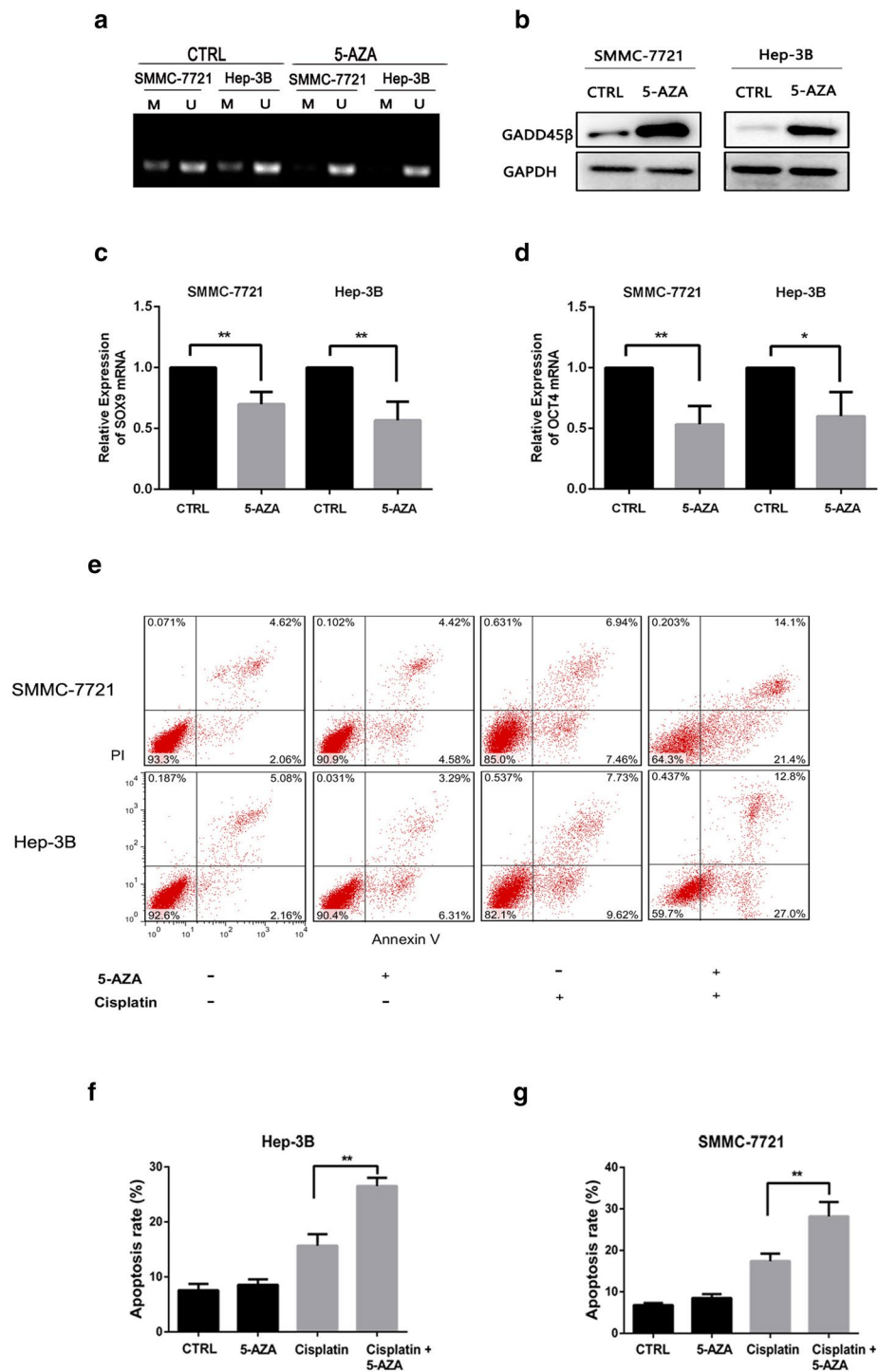


Fig. 4 Up-regulation of Gadd45β by 5-azacytidine increased the chemosensitivity of liver cancer cells through decreasing the stemness of HCC cells. **a** After SMMC-7721 and Hep-3B cells were treated with 5-azacytidine, the methylation-specific PCR (MSP) assay showed that methylation of Gadd45β promoter was obviously inhibited. **b** Western blot assay confirmed that the cells treated with 5-azacytidine promoted the expression of Gadd45β protein. **c, d** RT PCR then indicated that 5-azacytidine inhibited the mRNA expression of SOX9 and OCT4 in SMMC-7721 and Hep-3B cells. GAPDH was used as an internal reference (*P < 0.05; **P < 0.01). **e** Flow cytometry was then used to detect the apoptosis of cells. the result showed that 5-azacytidine treatment significantly enhanced the cisplatin-induced the apoptosis of SMMC-7721 and Hep-3B cells than control group. **f, g** Quantification of the apoptosis of cancer cells. Data are presented as the mean ± SD (**P < 0.01)

most studies in decreased expression of Gadd45 family is Gadd45a and Gadd45 g, and the main mechanism implicated in their decreased expression was due to the promoter methylation, such as many studies showed the frequent occurring of Gadd45a and Gadd45 g promoter methylation in many types of cancers [7, 14, 23, 24]. Besides the promoter methylation, the activated NF- κ B also was suggested to result in the repression of Gadd45 a and Gadd45 g in cancers [25]. Moreover, because Gadd45a is the downstream mediator of p53, thereby many studies showed the p53 status was associated with expression of Gadd45a in many cancers [26, 27]. In case of Gadd45 β , the promoter methylation is the most possible mechanism inducing the down-expression of Gadd45 β . Besides, it was reported that the transforming growth factor (TGF)-B can induce Gadd45 β expression [10]. In this study, it was found that Gadd45 β methylation was correlated with their expression in HCC tissues and liver cancer cell lines.

Conclusions

the expression of Gadd45 β was repressed in HCC tissues and liver cancer cells, and the Gadd45 β methylation may influence the expression. Furthermore, Gadd45 β inhibited the stemness properties of liver cancer cells, enhancing the chemosensitivity of liver cancer cells. This finding help to further clarify the importance of Gadd45 β in the progression of human HCC.

Abbreviations

HCC: hepatocellular carcinoma; Gadd45 β : growth arrest DNA damage-inducible gene 45 β ; IHC: immunohistochemistry; MSP: methylation-specific PCR; 5-AZA: 5-azacytidine.

Authors' contributions

XH and QZ: design, data analysis and interpretation, manuscript writing. YJ, ZH, XY, YZ: data acquisition and collection of samples. LW: data analysis, interpretation, financial support. FX: design, data analysis and interpretation, final approval of manuscript. BZ: collection of samples. All authors read and approved the final manuscript.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Informed consent was obtained from each patient under a protocol approved by the Hospital Research Ethics Committees.

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