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Original Article

The urea molecule 25-hydroxy vitamin D binding protein acts as a potential biomarker for liver cancer

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Abstract: Biological marker is of critical value for liver cancer diagnosis. 25-hydroxyl vitamin D binding protein (25-HVDP) regulates cancer and inflammatory response. This study thus investigated its potential value in the diagnosis of the early phase of liver cancer. Total of 156 liver cancer patients were recruited in parallel with 69 healthy volunteers for taking cisplatin and fluorouracil for 8 consecutive weeks. Liver cancer mouse model was generated. Real Time-PCR quantified 25-hydroxyl vitamin D binding protein level in patients and model mice to analyze its correlation with liver cancer, in order to investigate its value in the diagnosis of the early phase of liver cancer. Liver cancer patients had higher urea 25-hydroxyl vitamin D binding protein mRNA level than healthy volunteers. After treatment, mRNA of 25-hydroxyl vitamin D binding protein was decreased, whilst healthy volunteer had no significant difference (P = 0.23). Severe liver cancer patients had a higher urea 25-hydroxyl vitamin D binding protein than normal liver cancer patients (P = 0.0023). The level of urea 25-hydroxyl vitamin D binding protein was positively correlated with the liver cancer severity (P = 0.014). Liver cancer mice had a higher urea 25-hydroxyl vitamin D binding protein level than normal mice (P = 0.031). Urea 25-hydroxyl vitamin D binding protein may be a potential biological marker for liver cancer in both patients and model mice.

Keywords: Liver cancer, urea molecules, 25-hydroxyl vitamin D binding protein, diagnosis

Introduction

Liver cancer is one malignant tumor of the digestive system with about 0.01% overall incidence [1]. Complicated reasons are responsible for liver cancer pathogenesis, although current studies agree that viral infection is the major determining factor [2]. Liver cancer severely threatens patient's life quality and lifespan [3, 4]. The optimization of individualized treatment plan is one good solution [5-7], in which molecular targeted treatment has become a major research focus for liver cancer treatment [8-10]. Currently, both clinicians and scientists are working on the establishment of biomarkers with satisfactory specificity and sensitivity of liver cancer, for early phase diagnosis, treatment, and prognosis prediction of disease.

Early biomarkers being studied for liver cancer diagnosis mainly include tissue perfusion index, organ function parameters, inflammatory factors, and urea dynamic index, which, however, do not have satisfactory specificity [9, 10] or test sensitivity. Therefore, it is urgent in clinic to find better biomarker molecules for liver cancer [11]. Liver cancer involves multiple mechanisms, of which inflammatory mediator induced mitochondrial structural and functional change is one important factor [12, 13]. It is speculated that mitochondrial energy metabolism and self change of dynamic has certain correlations [14, 15]. 25-hydroxyl vitamin D binding protein is a proton transporter on mitochondrial membrane [16], and has multiple functions [17, 18]. This study aimed to investigate possible correlation between urea 25-hydroxyl vitamin D binding protein level in liver cancer patients and disease conditions. There has not been definitive evidence showing relationship between 25-hydroxyl vitamin D binding protein and liver cancer [19]. This study recruited liver cancer patients as the research object for analyzing the

correlation between 25-hydroxyl vitamin D binding protein level and liver cancer.

Patients and methods

Research objects (patients and mouse model)

A total of 156 liver cancer patients, including 69 normal liver cancer patients and 87 severe liver cancer patients, were recruited from Fuling center hospital, Shandong University from August 2013 to August 2017, in parallel with 69 healthy volunteers as the control group. Urea samples were collected from both liver cancer patients and healthy volunteers before and after treatment.

Inclusion and exclusion of liver cancer patients follow the criteria stipulated by American College of Liver Physicians (ACCP) of Society of Critical Care Medicine (SCCM) in 2016 [20, 21]. Inclusion criteria were as follows: 1) patients had not received radiofrequency ablation preoperatively; 2) Child-Pugh A/B; and 3) no concurrent cancers. Exclusion criteria were as follows: 1) recurrent HCC; 2) surgery-related death within 30 days after surgery; 3) extrahepatic metastasis; and 4) incomplete follow-up data. All liver cancer patients were divided into liver cancer patients with general conditions and severe liver cancer patients using previously documented criteria [22, 23].

Following common method, mouse liver cancer model was generated by diethylnitrosamine induction [24]. This study has been approved by the Animal Welfare Committee of our hospital. Model mice were treated with cisplatin and fluorouracil and were divided into blank control (I) group, cancer model (II) group, empty drug control for cisplatin (III) group, cisplatin treatment (IV) group, empty drug control for fluorouracil (V) group and fluorouracil treatment (VI) group. The golden standard for liver cancer diagnosis, alpha-fetoglobulin (AFP) was used to evaluate treatment efficacy among groups.

Urea sample preparation

Total of 10 ml urea samples were collected from all fasted patients in the morning. Urea samples were gently mixed and centrifuged at 150 g for 8 min. The supernatant was collected and stored in -70°C fridge (Dingguo Biotechnology, Beijing, China) for further use [17].

Real Time-PCR (RT-PCR)

Total RNA was extracted from urea samples following routine methods. Following the instruction of test kit (Beyotime Biotechnology, Shanghai, China), reverse transcription Real Time-PCR was performed [23]. In brief, urea samples were centrifuged at 800 r/min for 8 min. The supernatant was collected for extracting total RNA. Using RNA as the template and polyA primers, reverse transcription was performed at 42°C for 60 min. The primers were listed as the followings: 25-HVDP, forwards: 5'-GGAAGCTTTGGAGAGCTG AA-3', reverse: 5'-GCCATAAGTCCAACCAGGAA-3', actin: forwards: 5'-CGAGAA GATGACCCAGATCA-3', reverse: 5'-GATCTTCATGAGGTAGTCAG-3'. PCR was performed in a system containing 1 μl of complementary DNA (cDNA) template, 2 μl forward primer, 2 μl reverse primer, 4 μl dNTP mixture, 2 μl reaction buffer, and 1 μl Taq polymerase. PCR conditions were: 94°C for 5 min, followed by 30 cycles each consisting 94°C for 30 s, and 55°C for 30 s.

Western blot assay

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following routine methods (Dingguo Biotechnology, Beijing, China). In brief, urea samples were collected and quantified by bicinchoninic acid (BCA) protein quantification kit (Beyotime Biotechnology, Shanghai, China). Electrophoresis was performed followed by membrane transfer and primary antibody incubation (Rabbit anti-human 25-hydroxyl vitamin D binding protein antibody, 1:1000 dilutions, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Horse radish peroxidase (HRP) conjugated secondary antibody (Goat anti-rabbit secondary antibody, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubated for 1 h at room temperature incubation. Protein expression level was then analyzed.

Statistical analysis

All data were analyzed by SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Before analysis, all measurement data were firstly tested for normality. Those fitted normal distribution were presented as mean ± standard error of means (SEM). The Student's *t*-test was used to com-

Table 1. General information of patients and controls

	Patients group	Control group	*P* value
n	156	69	
M/F	70/86	36/33	0.312
Age	55 (17-79)	58 (16-81)	0.038

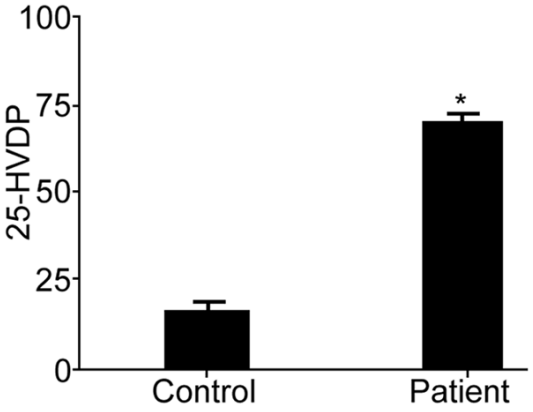

Figure 1. Real Time-PCR results of 25-hydroxyl vitamin D binding protein mRNA in urea samples of liver cancer patients. *$P < 0.05$, significant difference.

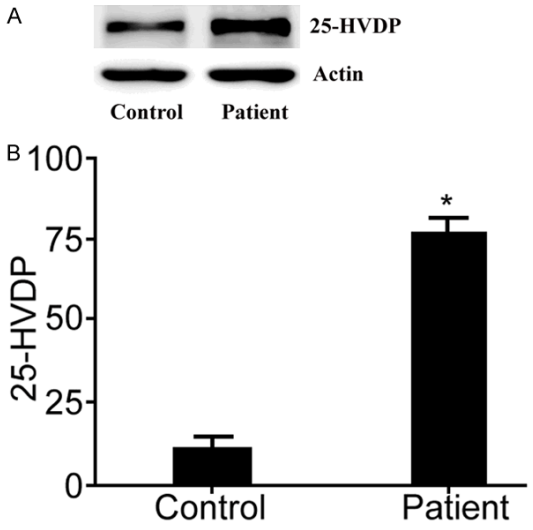

Figure 2. Western blot results for protein level of 25-hydroxyl vitamin D binding protein in liver cancer patients. *$P < 0.05$, significant difference.

pare the differences between two groups. The Tukey's post hoc test was used to validate the ANOVA for comparing measurement data among groups. Correlation analysis was performed using Pearson correlation. A significance was defined when $P < 0.05$.

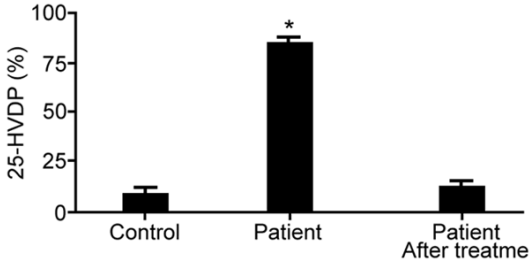

Figure 3. Decreased mRNA level of urea 25-hydroxyl vitamin D binding protein in liver cancer patients after treatment. *$P < 0.05$, significant difference.

Results

mRNA level of urea 25-hydroxyl vitamin D binding protein in liver cancer patients

Among all 156 liver cancer patients, averaged age was 55.3 ± 18.4 years (range: 17-79 years) (**Table 1**). Averaged age of healthy volunteers was 58.3 ± 17.4 years (range: 16-81 years). No significant difference existed in patient's age ($P = 0.038$) or gender ($P = 0.312$) between two groups (**Table 1**).

As shown in **Figure 1**, Real Time-PCR results showed significantly higher 25-hydroxy vitamin D binding protein (25-HVDP) mRNA level in urea of liver cancer patients compared to those in healthy volunteers ($P = 0.0017$).

Protein level of 25-hydroxyl vitamin D binding protein in liver cancer patients

As shown in **Figure 2**, Western blot showed significantly higher 25-hydroxyl vitamin D binding protein level in urea samples of liver cancer patients compared to those in healthy volunteers ($P = 0.0023$).

Decreased mRNA level in urea 25-hydroxyl vitamin D binding protein in liver cancer patients after treatment

As shown in **Figure 3**, after treatment, liver cancer patients had decreased urea 25-hydroxyl vitamin D binding protein mRNA levels.

Decreased protein level of urea 25-hydroxyl vitamin D binding protein in liver cancer patients after treatment

As shown in **Figure 4**, after treatment, protein level of urea 25-hydroxyl vitamin D binding pro-

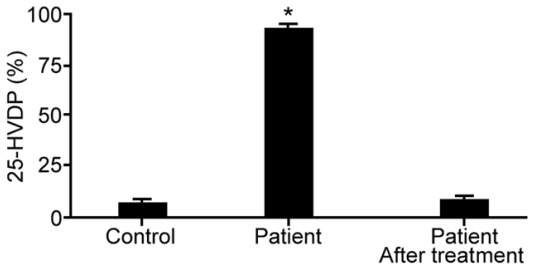

Figure 4. Decreased urea 25-hydroxyl vitamin D binding protein level in liver cancer patients. $^{*}P < 0.05$, significant difference.

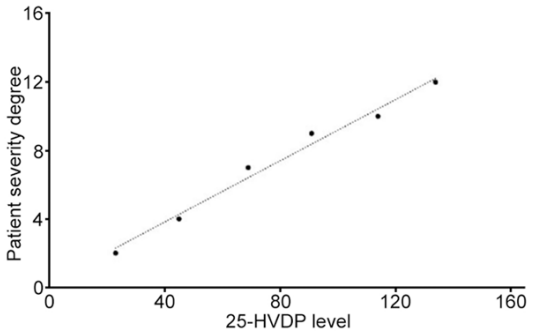

Figure 5. Significantly positive correlation between urea 25-hydroxyl vitamin D binding protein level and severity of liver cancer in patients.

tein was significantly decreased in liver cancer patients.

Significantly positive correlation between urea 25-hydroxyl vitamin D binding protein level and severity of liver cancer in patients

As shown in **Figure 5**, correlation analysis showed significantly positive correlation between 25-urea hydroxyl vitamin D binding protein level and severity of liver cancer disease.

Urea 25-hydroxyl vitamin D binding protein level in liver cancer mouse

To study possible role of 25-hydroxyl vitamin D binding protein in liver cancer model, we firstly generated mouse liver cancer model using diethylnitrosamine induction method. **Figure 6** showed successful generation of mouse model.

Liver cancer mice were treated with cisplatin or fluorouracil and divided into blank control (I) group, cancer model (II) group, empty drug control for cisplatin (III) group, cisplatin treatment (IV) group, empty drug control for fluorouracil (V) group and fluorouracil treatment (VI) group. The golden standard for liver cancer diagnosis, alpha-fetoglobulin (AFP) was used to evaluate treatment efficacy among groups. Results showed that after drug treatment, liver cancer model mice had disappearance of tumors. As shown in **Figure 6B** and **6C**, liver cancer mice had higher 25-hydroxyl vitamin D binding protein level than normal mice ($P = 0.031$). After drug treatment, liver cancer mice had normal urea 25-hydroxyl vitamin D binding protein level comparable to normal mice. Normal mice had unchanged 25-hydroxyl vitamin D binding protein level after drug treatment (data not shown).

Discussion

Early diagnosis and treatment are critical for liver cancer [25, 26]. It is still lack the effective biomarker for clinical testing of liver cancer [27]. This study thus investigated the possibility of urea 25-hydroxyl vitamin D binding protein as early diagnosis and prognosis prediction biomarker. Liver cancer is one inflammatory reaction process with highly complicated induction by various factors. During its onset and progression, various inflammatory factors cross-interact and accelerate to cause changes in body immune response status, forming one important reason for patient death [1, 2]. Based on current diagnostic criteria [20, 21], even under normal condition of liver cancer, patient has strong body immune response and receives severe threats for life health. Therefore, it is necessary to use more reasonable test measures for early screening and prognosis prediction of liver cancer. Nowadays, identification and functional study of early diagnostic marker for liver cancer has become a research focus and major challenge.

Over expression of urea molecules of 25-hydroxyl vitamin D binding protein can significantly decrease mitochondrial membrane potential. Under patho-physiological status, oxidative stress can damage hepatocytes with over-expression of urea molecules of 25-hydryxly vitamin D binding protein. However, few studies have been performed so far using urea 25-hydroxyl vitamin D binding protein as early diagnostic biomarker for liver cancer. We thus investigated this issue and found great difference between lipid and protein levels in urea samples, possibly affecting urea RNA extrac-

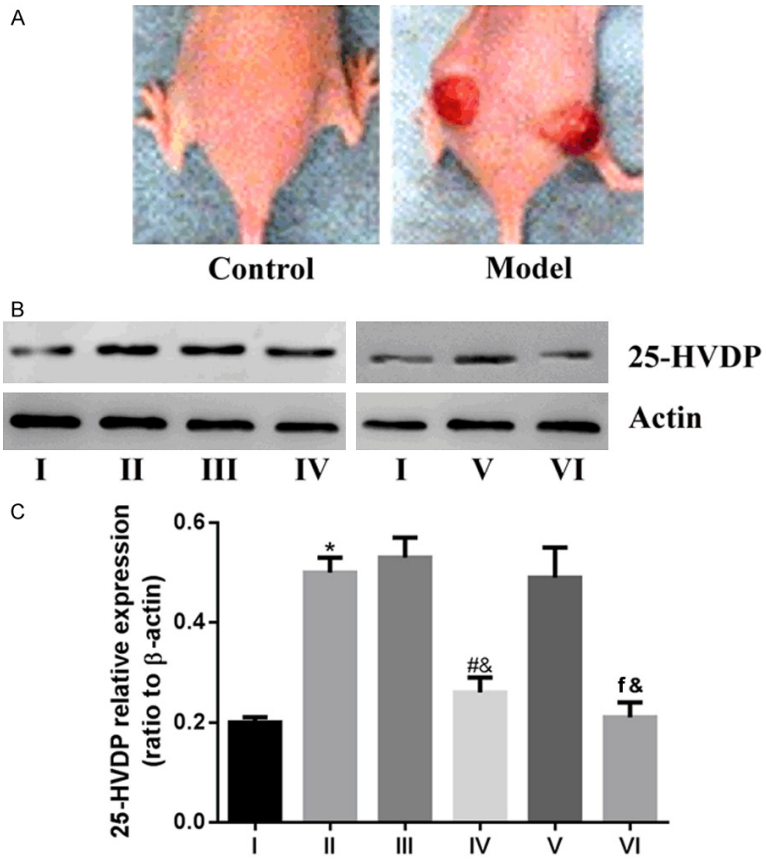

Figure 6. Western blot results for urea 25-hydroxyl vitamin D binding protein in liver cancer mice. Liver cancer mice were treated by cisplatin or fluorouracil and divided into blank control (I) group, cancer model (II) group, empty drug control for cisplatin (III) group, cisplatin treatment (IV) group, empty drug control for fluorouracil (V) group and fluorouracil treatment (VI) group. $^{*}P < 0.05$, significant difference. A: Liver cancer model; B: 25-HVDP expression; C: Quantitative expression of 25-HVDP. Compared with I, $^{*}P < 0.05$; Compared with III, $^{\#}P < 0.05$; Compared with V, $^{f}P < 0.05$; Compared with I, $^{\&}P > 0.05$.

tion. Of note, the extraction of RNA process may also introduce PCR reaction inhibitor. To eliminate such effects, we added nonsense microRNA sequence into our RNA samples as inhibitor for competing PCR, in order to eliminate interference to maximal extents. Therefore, methodology of this study is reliable and reasonable. Currently no literature has been reported for 25-hydroxyl vitamin D binding protein as a potential marker for liver cancer, making it one novelty of this study. Moreover, 25-hydroxyl vitamin D binding protein has significant advantages over previous markers including high sensitivity, safer sampling approach and no need for blood drawing. 25-hydroxyl vitamin D binding protein showed expressional change in both healthy population and liver cancer patients, indicating certain clinical significance.

Major findings of this study include: first, liver cancer patients had significantly higher urea 25-hydroxyl vitamin D binding protein mRNA and protein levels than those in healthy volunteers with significant difference ($P < 0.05$). Second, after treatment, liver cancer patients had lower mRNA and protein level of urea 25-hydroxyl vitamin D binding protein, with comparability to those in healthy volunteers. Third, severe liver cancer patients had higher urea 25-hydroxyl vitamin D binding protein level than those in normal liver cancer patients with significant difference ($P < 0.05$). These results showed that urea 25-hydroxyl vitamin D binding protein in liver cancer patients may be a specific biomarker for liver cancer. Protein level of urea 25-hydroxyl vitamin D binding protein was positively correlated with severity of liver cancer. Some studies have been performed using mouse liver cancer model and showed that abundant production of reactive oxygen species (ROS) of mice constitutively activates inflammatory pathways including plasma factors [14]. In addition, when intracellular ROS level was increased, inflammatory factor expression was subsequently upregulated [15, 16]. These findings showed the involvement of inflammatory factors in liver cancer as potential treatment targets. However, whether urea 25-hydroxyl vitamin D binding protein is correlated with this pathway requires further investigations. Weakness existed in this study, including: first, the relatively small sample size, and further study should increase sample size to study the possibility of urea 25-hydroxyl vitamin D binding protein for early diagnosis of liver cancer. Second, due to most liver cancer patients in clinics received chemotherapy or other treatment [28], whether the level of urea 25-hydroxyl vitamin D binding protein is under the influence of treatment requires further study. Third, this study lacked liver cancer rat model using

urea 25-hydroxyl vitamin D binding protein as the target to further substantiate conclusion of this study.

Conclusions

This study showed that urea 25-hydryxol vitamin D binding protein in liver cancer patients might represent a specific biomarker for liver cancer. Urea 25-hydroxyl vitamin D binding protein level is closely and positively correlated with the severity of liver cancer.

Disclosure of conflict of interest

None.

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