

Correlation between liver cancer pain and the HIF-1 and VEGF expression levels

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Abstract. A possible correlation between liver cancer pain and the hypoxia-inducible factor (HIF)-1 and vascular endothelial growth factor (VEGF) expression levels was examined. From January, 2015 to January, 2016, 30 patients suffering from liver cancer with pain, 30 patients with liver cancer without pain and 30 hepatitis patients with pain were enrolled in the study. Pain level was evaluated by visual analogue scale (VAS), the expression levels of HIF-1 and VEGF mRNA were determined by RT-PCR and the expression levels of HIF-1 and VEGF proteins were examined by ELISA. Before intervention, the VAS in the hepatitis group was significantly higher than that of the liver cancer pain group. However, after intervention the VAS in the two groups was reduced. HIF-1 and VEGF mRNA expression levels in the liver cancer pain group were significantly higher than those in the liver cancer group before and after intervention. The expression levels of HIF-1 and VEGF mRNA in the hepatitis group were the lowest. The expression levels of HIF-1 and VEGF mRNA in the liver cancer pain group considerably increased after intervention. The expression levels of HIF-1 and VEGF mRNA in the other two groups showed no changes before or after intervention. Before and after the intervention, VAS in the liver cancer pain group was positively correlated to the expression levels of HIF-1 and VEGF. Thus, pain occurrence and the pain level in liver cancer patients were correlated with the expression levels of HIF-1 and VEGF. As the regular three-step medicine analgesic ladder is ineffective in these cases, verification of HIF-1 and VEGF expression levels may be considered the new target for pain release.

Introduction

The incidence of liver cancer pain is approximately 30-70% (1). This pain primarily happens during the middle

or terminal stages and negatively influences the therapeutic effects, while seriously reducing quality of life of patients. The occurrence of pain is a complex physical and psychological process produced by noxious stimulation on the body and the body's response to pain through corresponding physical movement and vegetative visceral reaction. Occurrence of cancer pain is an incompletely understood phenomenon, however, it was shown that the mechanism involved in the cancer pain occurrence was influenced by several parameters. Parameters such as: i) Release of a nociceptive substance by tumor cell that acts on peripheral nerve (5-hydroxytryptamine, histamine and bradykinin); ii) tumor infiltration into nerves and vessels, which causes sharp neuralgia emitting to nerves distributed on body surface and ischemic pain led by vessel blockages; and iii) tumor metastasis to bones, spinal cord, lymph and other organs (2,3).

Psychological factors such as disturbance, anger and depression also play important roles in the occurrence of pain (4). Certain effects were visualized by the three-step medicine analgesic ladder therapy for scientific pain release; however, the pain occurrence mechanism is not intervened specifically (5). The pain level was shown to be closely related to the growth of the tumor and its micro-environment (6-8). Hypoxia-inducible factor (HIF)-1 is an important transcriptional regulatory factor that mediates an adaptive response to the hypoxic micro-environment for tumor cells and acts on an important upstream regulatory protein that controls the neovascularization of tumor, maintains energy metabolism, and promotes cell proliferation, infiltration and metastasis (9). Vascular endothelial growth factor (VEGF) has shown the strongest effect on inducing tumor neovascularization with the highest specificity (10,11).

In order to find a new intervention target for pain release, we analyzed the possible correlation between liver cancer pain occurrence and HIF-1 and VEGF expression levels.

Materials and methods

Patients. From January, 2015 to January, 2016, 30 patients suffering from liver cancer with pain, 30 patients with liver cancer without pain and 30 hepatitis patients with hepatalgia were enrolled in the present study (they were selected by closest matching method, age- and gender-matched 1:1) and established as the hepatitis group. The liver cancer pain group included 16 males and 14 females, aged 48-76 years (average

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age, 62.3 ± 15.4 years). Five patients were in early stage while 8 were in middle or terminal stages. The liver cancer group included 17 males and 13 females, aged 45-78 years (average age, 62.5 ± 13.6 years). The liver cancer group comprised 8 patients in early stage and 22 in middle or terminal stages. The hepatitis group comprised 15 males and 15 females, aged 43-79 years (average age, 63.2 ± 13.6 years). In the hepatitis group, 8 patients were diagnosed with hepatitis B. The basic information of patients in all 3 groups was comparable.

Exclusion criteria for the study were: Patients treated with analgesic, hepatic surgeries, radiotherapy or chemoradiotherapy; patients who suffered from other pain causing diseases (including autoimmune diseases, osteoarticular diseases, and other neoplastic diseases); patients with a history of surgery or trauma; and those who had difficulties in identifying the level of pain because of hyperpathia.

The present study was approved by the Ethics Committee of the Cangzhou Central Hospital. Written informed consent was obtained from each patient.

Methods and observation indexes. Cancer patients were treated with the three-step medicine analgesic ladder and hepatitis patients with pain were treated with primary disease therapy. Symptomatic treatment was conducted for the two groups. Visual analogue scale (VAS) was applied for pain evaluation, RT-PCR was used for the determination of HIF-1 and VEGF mRNA expression levels and enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of HIF-1 and VEGF in the serum. VAS scores ranged from 0 to 10, where the higher scores were associated with more aggravated pain levels. ELISA results were read on an automatic multifunctional microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the instructions of the ELISA kit supplied by Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China) and the average score was taken.

RT-PCR testing. Key reagents and instruments used were: RNA extraction kit, reverse transcription kit, DNase and SYBR-Green PCR mixture from Sigma-Aldrich (St. Louis, MO, USA), ultraviolet spectrophotometer from Beijing Liuyi Instrument Factory (Beijing, China), and fluorescent quantitative PCR from Linegene Bori.

Key procedures involved: Total RNA was extracted using regular TRIzol (R&D Systems, Inc., Minneapolis, MN, USA). cDNA was reverse transcribed using the primers: HIF-1 forward, 5'-AGAACTCTCAGCCACAGTGC-3' and reverse, 5'-CTAGCAGAGTCAGGGCATCG-3', 219 bp; VEGF forward, 5'-ATTCAACGGACTCATCAGCCA-3' and reverse, 5'-GCAACCTCTCCAAACCGTTG-3', 150 bp; β -actin forward, 5'-CCCATCTATGAGGGTTACGC-3' and reverse, 5'-TTTAA TGTCACGCACGATTTC-3', 150 bp. For the reaction system 2X SYBR PCR mixture (10 μ l), primers (10 μ M, 1 μ l each), template (2 μ l) and volume was adjusted to 20 μ l with water. The reaction conditions were: 95°C for 10 min, 95°C for 15 sec and 59°C for 60 sec with 45 cycles. Solubility curves were obtained and the result was demonstrated by $2^{-\Delta\Delta Ct}$.

Statistical analysis. SPSS statistics 20.0 (Chicago, IL, USA) was used for statistical analyses. Measurement data are

Table I. Comparison of VAS before and after intervention.

Groups	Before intervention	1 month after intervention
Liver cancer	3.6 ± 1.0	3.0 ± 0.9
Hepatitis	5.4 ± 1.2	0.8 ± 0.3
t	6.302	6.758
P-value	0.026	0.020

VAS, visual analogue scale.

presented as mean \pm standard deviation, and comparison among several groups was analyzed by single-factor ANOVA. Comparison between 2 groups was analyzed by the independent sample t-test and comparison within the group was analyzed by paired U test. Enumeration data were presented as frequency or percentage and compared using the χ^2 test between groups. Correlative analysis of measurement data was conducted using the Pearson test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of VAS. VAS in the hepatitis group was significantly higher than that in the liver cancer pain group before intervention, but VAS significantly decreased after intervention. VAS in the two groups decreased after intervention. Differences were statistically significant ($P < 0.05$) (Table I).

Expression levels of HIF-1 and VEGF mRNA. Before intervention, the expression levels of HIF-1 and VEGF mRNA in the two liver cancer groups were higher than those in the hepatitis group. HIF-1 and VEGF mRNA levels in the liver cancer pain group were significantly higher than the levels observed in the liver cancer group before intervention. Differences were statistically significant ($P < 0.05$). The expression levels of HIF-1 and VEGF mRNA in the liver cancer pain group increased significantly after intervention and the differences were of statistical significance ($P < 0.05$). HIF-1 and VEGF mRNA levels in the liver cancer and hepatitis groups did not change significantly after intervention ($P > 0.05$) (Table II).

Expression levels of HIF-1 and VEGF. HIF-1 and VEGF levels in serum showed similar results as their corresponding mRNA levels (Table III).

Correlative analysis. VAS in the liver cancer pain group before and after the intervention was positively correlated with the expression levels of HIF-1 and VEGF ($P < 0.05$). Comparison of VAS and HIF-1 mRNA level before intervention produced the following data: $r = 0.326$, $P = 0.025$; $r = 0.421$, $P = 0.020$. Comparison of VAS and the expression level of VEGF before intervention produced the following data: $r = 0.352$, $P = 0.022$; $r = 0.457$, $P = 0.016$. Comparison of VAS and the expression level of HIF-1 after intervention: $r = 0.313$, $P = 0.027$; $r = 0.430$, $P = 0.022$. Comparison of VAS and the expression level of VEGF after intervention: $r = 0.339$, $P = 0.023$; $r = 0.448$, $P = 0.018$.

Table II. Expression levels of HIF-1 and VEGF mRNA in different groups.

Groups	HIF-1				VEGF			
	Before intervention	1 month after intervention	t	P-value	Before intervention	1 month after intervention	t	P-value
Liver cancer pain	0.3264±0.0425	0.4152±0.0362	8.532	0.006	0.4201±0.0725	0.4632±0.0936	7.659	0.012
Liver cancer	0.1235±0.0127	0.1028±0.0532	0.932	0.241	0.2316±0.0421	0.2423±0.0538	0.564	0.827
Hepatitis	0.0039±0.0010	0.0052±0.0023	0.632	0.527	0.0063±0.0021	0.0058±0.0030	0.125	0.632
F-value	10.632	15.624			13.623	14.521		
P-value	<0.001	<0.001			<0.001	<0.001		

HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

Table III. Expression level of HIF-1 and VEGF (pg/ml) before and after intervention.

Groups	HIF-1				VEGF			
	Before intervention	1 month after intervention	t	P-value	Before intervention	1 month after intervention	t	P-value
Liver cancer pain	13.2±2.6	16.3±3.0	6.598	0.033	16.3±3.2	18.5±3.6	7.120	0.026
Liver cancer	8.6±2.2	8.5±2.3	0.428	0.662	9.0±2.3	9.1±2.4	0.425	0.659
Hepatitis	1.3±0.4	1.0±0.3	0.128	0.754	1.8±0.6	1.6±0.5	0.230	0.548
F-value	12.634	17.201			16.230	18.330		
P-value	<0.001	<0.001			<0.001	<0.001		

HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

Comparison of VAS of hepatitis group had no correlation with HIF-1 and VEGF expression levels ($P>0.05$).

Discussion

The hypoxic micro-environment has been shown to be closely correlated with the invasion and metastasis of liver cancer cells (11). HIF-1 is a protein composed of the two subunits α and β . As the unique subunit functioning on oxygen condition, HIF-1 α has a positive correlation with the hypoxic level of the tumor. The expression level of HIF-1 increased with the severing of the hypoxic level. A relatively high level of HIF-1 α can be reached in a short period of time after the occurrence of hypoxia (12). Furthermore, HIF-1 also regulates the production of VEGF, angiopoietin-1, -2, PDGF-B and PLGF, and was involved in the entire process of angiogenesis (12). Invasion and metastasis have been shown to accelerate by the presence of HIF-1 through the promotion of cytoactive induction of matrix metalloproteinase, and influence on the expression of adhesion molecules (13,14). The direct influence on the expression of multi-drug resistance gene 1/P-glycoprotein by HIF-1 was regarded as one of the significant mechanisms leading to drug resistance. VEGF is a glycosylated multifunctional protein. VEGF level has been shown to correlate well with angiopoiesis of several types of tumors (15). VEGF is the first angiogenesis

factor ever found to be induced by hypoxic condition, and a significant target gene among those directly regulated by HIF-1. VEGF synthesis was affected by hypoxic condition through two main mechanisms: Hypoxic condition retards the degradation of VEGF mRNA and increases its stability. While VEGF is transcribed and activated by the mediation of HIF, the expression level of VEGF mRNA can be increased by a combination of hypoxic regulatory elements affecting the regulatory regions located at 3' and 5' NTR to upregulate mRNA transcription. VEGF cannot be produced in tumor cells and angiogenesis of the tumor may be inhibited if the HIF-1 gene is removed or HIF-1 transcription is interrupted (16,17).

Results obtained in the present study suggested that VAS in the hepatitis group was significantly higher than that in the liver cancer pain group before intervention, but decreased significantly after intervention. In addition, the VAS in both groups was decreased significantly after intervention. Liver cancer pain is most likely the chronic persistent dull pain, mechanically stretched on hepatic lobule capsule or hepatic capsule during the growth of tumor; while hepatitis pain is often accompanied with the release of inflammatory mediators, which peaked at the early stage and remitted with the healing of disease.

We showed that the HIF-1 and VEGF expression levels in the liver cancer pain group were significantly higher

than those in the liver cancer group before intervention. The HIF-1 and VEGF expression levels in liver cancer pain group surged after intervention. Attention should be paid to the fact that the increase observed in the HIF-1 and VEGF expression levels was well correlated with failed therapeutic efforts. Pain occurrence and the pain level increased with the surge in HIF-1 and VEGF expression levels. VAS in the liver cancer pain group before and after the intervention was positively correlated to HIF-1 and VEGF expression levels before and after intervention.

Thus, pain in liver cancer patients is correlated with the expression levels of HIF-1 and VEGF. As the regular three-step medicine analgesic ladder is ineffective in these cases, verification of HIF-1 and VEGF expression levels may be considered the new target for pain release.

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