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Effects of arsenic trioxide on the expression of ezrin in hepatocellular carcinoma

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Abstract

The aim of the study was to investigate the effects of arsenic trioxide (As_2O_3) treatment on the expression of ezrin and serum alpha-fetoprotein (AFP) levels in hepatocellular carcinoma (HCC).

A total of 24 patients (20 males and 4 females) with resectable HCC were treated with venous injection of As_2O_3 for 14 days (10 mg/d) before surgery. The ezrin expression and serum AFP levels were assessed before and after treatment, respectively.

The serum AFP levels were 325.5 ng/L before treatment and 278.6 ng/L after treatment, with statistical significant difference ($Z = -2.360$, $P < .05$). The expression of ezrin was negative, weak positive, and strong positive in 11, 7, and 6 cases, respectively, before As_2O_3 treatment, and 17, 5, 2 cases respectively after the treatment. The difference between the 2 groups was statistically significant ($\chi^2 = 5.619$, $P < .05$). Also, the results showed that there was a significant correlation between the high serum AFP level (AFP $\geq$ 500 ng/L) and high expression of ezrin ($\chi^2 = 8.080$, $P < .05$).

As_2O_3 treatment can significantly downregulate the expression of ezrin in HCC.

Abbreviations: AFP = serum alpha-fetoprotein, As_2O_3 = arsenic trioxide, COX-2 = cyclooxygenase-2, CT = computed tomography, EGFR = epidermal growth factor receptor, ELISA = enzyme-linked immunosorbent assay, HCC = hepatocellular carcinoma, PCNA = proliferating cell nuclear antigen, PLT = platelets, VEGF = vascular endothelial growth factor, WBC = white blood cell.

Keywords: alpha fetoprotein, arsenic trioxide, ezrin, gene expression, hepatocellular carcinoma

1. Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related deaths in China. In recent years, the HCC resection rate has been significantly improved because of the improvement in adjuvant therapy and surgical techniques. However, the 5-year survival rate is not significantly increased, due to the recurrence and metastasis. Therefore, the research upon HCC recurrence and metastasis has become one of the main topics for HCC treatment.[1] Considering the satisfactory outcomes of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia, the researches wonder if As_2O_3 has the same effect on HCC. Systemic and local infusions with As_2O_3 have been demonstrated to inhibit the tumor growth of HCC in clinic. Besides, As_2O_3 is an effective medicine in the treatment of lung metastases of HCC and cancer-related pain.[1–6] However, the anti-tumor mechanism of As_2O_3 is still unknown. Recent researches have shown that As_2O_3 can inhibit the invasion and metastasis of HCC cells through inhibiting RhoC expression.[6] RhoC and its downstream effector molecule ezrin are members of the Rho subfamily, which may be involved in antitumor function mediate by As_2O_3 in HCCs. But more evidence is needed to demonstrate the hypothesis. This article aimed to explain the mechanism of the inhibition of HCC cells metastasis by As_2O_3 through evaluating the expression patterns of ezin before and after As_2O_3 treatment.

2. Materials and methods

2.1. Study patients

The patients included in this study should meet the following criteria: (1) the patients were diagnosed with HCC according to the clinical diagnosis, dynamic computed tomography (CT) or magnetic resonance imaging, and pathological diagnosis; (2) Karnofsky performance scale (KPS) $\geq$ 90; (3) *white blood cell* (WBC) $\geq 4.0 \times 10^9$/L; (4) (PLT) $\geq 80 \times 10^9$/L; (5) normal prothrombin time; (6) Child A; (7) suitable for surgical treatment. The patients would be excluded from the study, if any of the following conditions appeared: (1) arterioportal fistulas (APFs); (2) portal vein and/or inferior vena cava tumor thrombus; (3) distant metastasis.

According to the selection criteria, from March 2013 to April 2014, 24 patients aged 42 to 74 years (mean aged 58.6 ± 8.6 years) who were diagnosed with resectable HCC were selected in this research. Among them, 10 patients (41.7%) were diagnosed with massive tumors, whereas the rest with nodular type (58.3%). This research was reviewed and approved by the Ethic Committee of Navy General Hospital of PLA, and the informed consent was signed by each participant.

3. Methods

3.1. As_2O_3 treatment

Each patient was administered an intravenous infusion of As_2O_3 (Jia Tong Pharmaceutical Company, Heilongjiang, China)

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10 mg/d for 14 days. The resection operation was performed for the HCC cases at 3 to 7 days after the As_2O_3 treatment. The hepatic segmentectomy was performed for 12 patients (I and II: 6; III and IV: 6) according to the Glissonean pedicle transection method: 5 patients received hepatic left lateral lobectomy, whereas 7 patients were treated with half hepatectomy (left: 1; right: 6).

3.2. AFP detection

Primary antibody for AFP was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Dallas), and the UltraSensitiveTM SP kit was purchased from Fuzhou Maixin Biotechnology (Fuzhou, China). Enzyme-linked immunosorbent assay (ELISA) was applied to detect the serum AFP levels according to the instructions. In brief, the serum samples collected from the patients before and after As_2O_3 treatment were placed into the polystyrene reaction plate microporous coated with AFP antibody. Then, the color reactions were observed after the enzyme substrates added, and the stop buffer was used to terminate the reaction. Then, the serum AFP levels were detected and recorded.

3.3. Ezrin gene detection

The tissue specimens obtained before the As_2O_3 treatment or the resection afterwards were preserved in the 10% formalin solution. The expression of ezrin protein was assessed by the SP immunohistochemical method using the antihuman Ezrin protein. The immunohistochemical staining was performed according to the UltraSensitiveTM SP kit manual. Positive staining was identified as brown granules for both the carcinoma cells and interstitial cells, with the compartment without cells being used as the blank background control. The level of staining was represented by color scoring after background subtraction: 0 for colorless, 1 for faint yellow, 2 for pale brown, and 3 for brown. The percentage of positive cells in total number of cells was calculated from observations from 5 randomly chosen visual fields under high power (×400) for each slide, which was then scored as 0 for negative staining, 1 for the percentage $\leq$ 29%, 2 for 30% to 69%, and 3 for 70% to 100%. The level of positivity was then represented by the product of scores of the level of staining and the percentage of positive cells: 0 for no expression, 1–4 for weak expression, and 6–9 for strong expression. The expression levels and distributions of ezrin gene in both the tumor tissues and the adjacent tissues were analyzed, and compared.

3.4. Statistical analysis

The difference of serum AFP levels was compared with the rank sum test, whereas the changes of ezrin gene expression were evaluated by rating the information chi-square test. The chi-square test was performed to estimate the relationship between serum AFP levels and ezrin gene expression. $P < .05$ was considered statistically significant.

4. Results

4.1. Serum AFP changes before and after treatment

The serum AFP level before the As_2O_3 treatment ranged from 7.5 to 3000.0 (median: 325.5) ng/L, and the level after the treatment ranged from 4.5 to 3000.0 (median: 78.6) ng/L. The difference of AFP level before and after treatment was statistically significant ($Z = -2.360$, $P < .05$).

Table 1

Correlation analysis between the high AFP level and ezrin expression strength.

	AFP levels, ng/L		
Ezrin	<500	≥500	Sum
Strong	2	6	8
Weak	7	5	12
None	22	6	28
Sum	31	17	48

AFP = serum alpha-fetoprotein.
$\chi^2 = 8.080$, $P < .05$.

4.2. Ezrin protein expression level before and after treatment

The ezrin protein expression level before and after treatment in HCC tumor tissues are illustrated in Table 1. Before the treatment with As_2O_3, a total of 13 patients with ezrin positive expression were detected in the 24 patients (54.17%), of which 6 cases were strongly expressed, and 7 cases were weakly expressed. After the treatment, only 7 patients were detected to be ezrin positive expression (29.17%); among them, there were 2 cases with strong expression, and 5 cases with weak expression. The difference before and after treatment was statistically significant ($\chi^2 = 5.619$, $P < .05$) (Fig. 1A–D)

4.3. Correlation analysis between serum AFP and ezrin gene expression

The serum AFP and ezrin expression levels were analyzed before and after the treatment. The cumulative total number of 31 cases were with AFP < 500 ng/L, and their expression of ezrin graded as strong, weak, and no expression was 2, 7, and 22 cases, respectively. Although in cumulative 17 cases with AFP ≥ 500 ng/L, the ezrin expression graded as strong, weak, and no expression was 6, 5, 6 cases, respectively. The results showed that the AFP levels were significantly associated with the ezrin expression level ($\chi^2 = 8.080$, $P < .05$) (Table 1).

Besides, 3 months after operation, all the patients had a physical review. No portal vein thrombosis or recurrence was observed.

5. Discussion

Accumulating clinical data showed that the ezrin expression is involved in the modification of the cell morphology, membrane structure formation, cellular movements, and adhesion-related signal pathway. What is more, it plays an important role in tumor cell motility, adhesion, and invasion activities. Its expression level is closely related to HCC cell development, metastasis, and prognosis. The positive expression rate of ezrin in HCC was significantly higher than that in adjacent tissues, liver cirrhosis, and normal liver tissues. Also, the positive expression of ezrin was obviously correlated with poor differentiation. Meanwhile, the tumor size and metastasizing tendency seemed to be strongly related with ezrin expressions. The enhanced expression of ezrin was observed in the tissues with tumor size greater than 3 cm and metastatic tissues.[7] As_2O_3 could inhibit the tumor proliferation and angiogenesis through multiple antineoplastic mechanisms, including downregulating cyclinD1, proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), integrin β1, epidermal growth factor receptor (EGFR), RhoC expression, as well as up-regulating the

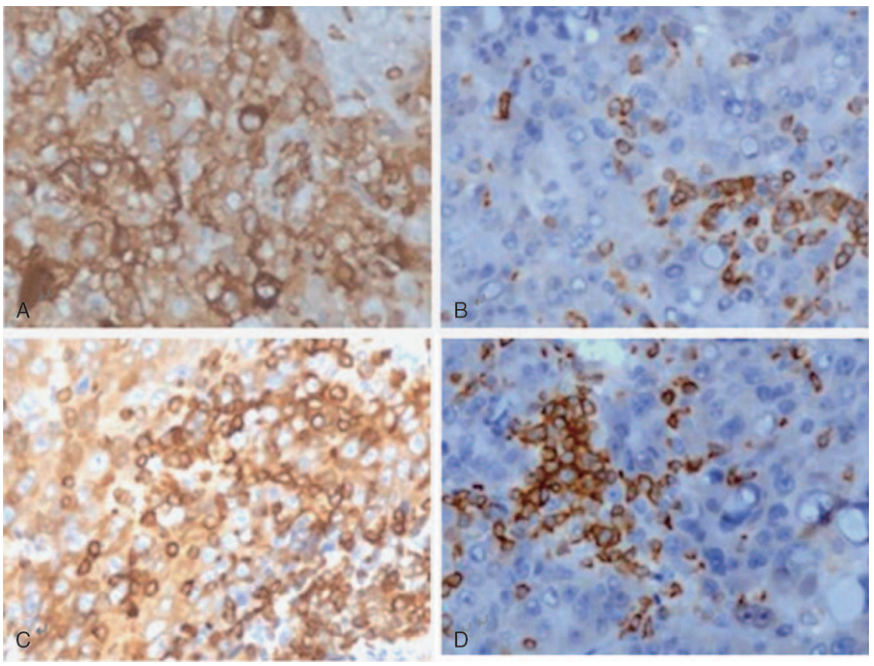

Figure 1. Immunohistochemical analysis of Ezrin expression in HCC before and after As_2O_3 treatment. (A) Brown granules were observed in more than 70% of HCC cells and interstitial cells cytoplasm in the sample; before As_2O_3 treatment, expression of Ezrin is strong. (B) After the As_2O_3 treatment, Brown granules were observed in less than 50% of HCC cells cytoplasm; weak expression of Ezrin is shown. (C) Brown granules expressed by the ezrin gene were observed in more than 90% of round-shaped HCC cells (probably the tumor stem cell) in the sample; before As_2O_3 treatment, expression of Ezrin is strong. (D) Brown granules were observed in less than 30% of HCC cells; after the As_2O_3 treatment and round-shaped HCC cells could merely be found, weak expression of Ezrin is demonstrated. As_2O_3 = arsenic trioxide, HCC = hepatocellular carcinoma.

expression of E-cadherin. Additionally, it could induce cell apoptosis through enhancing bax expression and downregulating bcl-2/bax.[3,4] Chan et al[8] had demonstrated that As_2O_3 could induce hepatoma cell apoptosis of multidrug-resistant strains of R-HepG2. Given its functional roles in tumorigenesis, As_2O_3 was confirmed as an effective drug in treatment of advanced HCC. It could prolong the survival of patients with advanced HCC, and effectively relieve the symptom of abdominal pain. Kim et al[5] performed transcatheter artery chemoembolization with the mixture of arsenic trioxide and Lipiodol; the result showed that the mixture significantly inhibited the growth of HCC tumor, whereas it did not increase the liver and kidney toxicity, and there was a positive correlation between the tumor inhibition rate and the dose of arsenic trioxide. Griffin et al[9] demonstrated that As_2O_3 could selectively inhibit tumor angiogenesis, thereby enhancing the efficacy of hyperthermia and radiation therapy of solid tumors. Liver cancer stem cells research has become a hot topic, how to kill liver cancer stem cells is key to the ultimate control and cure of liver cancer. In our study, a large number of small round cells were found in the cancer tissue sections of 1 patient before using As_2O_3; they are suspected HCC stem cells. Small round cells disappeared after As_2O_3 treatment. So it was presumed that As_2O_3 is associated with the clearance of HCC stem cells.

Sell and Leffert et al[10] found that As_2O_3 could induce the apoptosis of HCC stem cells through targeting promyelocytic leukemia protein to control tumor growth, inhibit tumor invasion and metastasis, and prevent recurrence. As_2O_3 can reinforce the anti-tumor effect of other chemotherapeutic agents (cisplatin, adriamycin). Wang et al[11] had proved that in combination with As_2O_3, the anti-tumor effects of cisplatin were significantly improved, and the As_2O_3 concentration was positively correlated with the tumor inhibition rate. Recent study[12] showed 5-FU combined with As_2O_3 could significantly enhance the inhibitory effect on liver cancer in nude mice. As_2O_3 was resumed to have enhanced inhibition of cell proliferation and induction of apoptosis, and no obvious side effects of combination therapy was found.

The molecular mechanisms of treatment of malignant tumors using As_2O_3 remains to be further elucidated. Many studies had showed As_2O_3 could suppress RhoC expression[6] and thus inhibit the expression of ezrin which was the downstream effector molecule of Rho, leading to inhibit the HCC progression and metastasis.[13] RhoC and ezrin gene expression have a close relationship with HCC recurrence, invasion, metastasis, and the formation of portal vein thrombosis. The expression of RhoC and ezrin in highly aggressive cancer tissues and tumor thrombi was significantly higher than that in low invasive and noncancerous tissue.[6,12–15] Chen et al[16] found that metastasis of liver cancer can be inhibited by blocking the Rho-kinase-mediated phosphated ezrin protein. These results indicated that As_2O_3 may regulate the expression of RhoC signaling molecules and ezrin, thus inhibiting liver cancer cell growth, invasion, and metastasis. However, more direct evidence will be required to support the hypothesis.

In conclusion, in this research, a group of resectable HCC patients received the As_2O_3 treatment 2 weeks before the surgeries, and the ezrin expressions in HCC tissue were obtained before the treatment of As_2O_3 and after the excision. The result showed that (1) ezrin gene expression was significantly decreased after As_2O_3 treatment, indicating that As_2O_3 can significantly reduce the ezrin gene expression; (2) As_2O_3 therapy can significantly decrease serum AFP levels that further illustrate the ability of As_2O_3 to inhibit the growth of tumor cells. Our

study shows that As_2O_3 is an effective drug in HCC treatment, which can affect the Rho signaling pathway, and perhaps thereby inhibiting the tumor growth, recurrence, invasion, and the formation of portal vein thrombosis, which need to be further studied in future; (3) the high levels of AFP are significantly correlated with the ezrin expression.

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