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Original Article

Effect of autophagy inhibitor 3-methyladenine combined with TP chemotherapy on nasopharyngeal carcinoma and EGFR and VEGF levels in tissues

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Abstract: Objective: Explore the effects of 3-methyladenine (3-MA) combined with docetaxel and cisplatin (TP) chemotherapy regimen on the treatment of nasopharyngeal carcinoma and expression levels of EGFR and VEGF in tissues from patients with nasopharyngeal carcinoma. Methods: A retrospective analysis was conducted on 249 patients with nasopharyngeal carcinoma, 134 patients received single TP chemotherapy after admission were grouped as the control group and 115 patients treated with 3-MA combined with TP chemotherapy were considered as the study group. The expression of EGFR and VEGF in tissues was detected by Western blot. The difference in efficacy between the two groups was compared and the survival rate of the patients was recorded by a 5-year follow-up of prognosis. Results: The treatment efficacy of 90.43% reported in the study group was significantly higher than that observed for the control group ($P < 0.010$). The incidence of adverse reactions was 13.01% in the study group and was significantly lower than that in the control group ($P < 0.010$). EGFR and VEGF levels in the study group were significantly lower than those in the control group at 15 and 30 days after treatment ($P < 0.010$). The 5-year survival rate was significantly higher in the study group than in the control group ($P = 0.010$). Conclusion: 3-MA combined with TP chemotherapy was significantly superior to single TP regimen for the treatment of nasopharyngeal carcinoma and inhibited the expressions of VEGF and EGFR in patient tissues.

Keywords: 3-MA, TP chemotherapy, nasopharyngeal carcinoma, EGFR, VEGF

Introduction

Nasopharyngeal carcinoma, a malignant cancer located at the nasopharyngeal site, is a common form of cancer [1]. At present, more than 1.8 million people suffer from nasopharyngeal cancer worldwide, and a rising trend in this number has been observed [2]. The incidence of nasopharyngeal cancer is strongly associated with regional and ethnic differences. According to the statistics of Sun et al. [3], the incidence of nasopharyngeal cancer in yellow species is about 3.5 times higher than that reported in other races. In some extremely populated countries (China and India), the number of patients suffering from nasopharyngeal cancer is more than eight times the number observed in other countries [4]. As the distant metastasis rate of nasopharyngeal carcinoma is as high as 20% to 40%, the treatment possibilities are greatly decreased following invasion and metastasis [5]. The high incidence and mortality rates of nasopharyngeal carcinoma demand effective measures for improved prognosis of patients. The continuous improvement in research and the development of medical technology have led to an increase in the treatment efficiency of radiotherapy and chemotherapy for nasopharyngeal carcinoma by 80% in clinical settings. The survival rate of patients after treatment has been stable at about 70% [6]. However, some patients exhibit local tumor recurrence and distant metastasis, resulting in poor prognosis [7]. Therefore, efforts have been directed to improve the sensitivity of radiotherapy and chemotherapy in patients

with nasopharyngeal carcinoma for better prognosis.

Several recent studies [8-10] have highlighted the application of the combination of the autophagy inhibitor 3-methyladenine (3-MA) and radiochemotherapy in the diagnosis and treatment of cancer. This combination is known to effectively reduce the proliferation and induce apoptosis of cancer cells. However, very few reports are available on the application of this strategy in nasopharyngeal carcinoma. Since 2010, our hospital has used 3-MA for the treatment of patients with nasopharyngeal cancer, and relatively effective therapeutic outcomes have been observed. Therefore, we retrospectively analyzed patients with nasopharyngeal carcinoma that underwent 3-MA combined with radiochemotherapy in our hospital from 2010 to 2013 and evaluated the expression levels of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) in these patients during the course of treatment, with an aim to provide a reference and guidance for future clinical diagnosis and treatment of nasopharyngeal carcinoma.

Materials and methods

This study has been approved by the Ethics Committee of The Second Affiliated Hospital of Shaanxi University of traditional Chinese Medicine. All study participants had given their written informed consent before participating in the study.

Research subjects

A retrospective analysis of patients admitted to Second Affiliated Hospital of Shaanxi University of traditional Chinese Medicine from May 2010 to May 2013 was performed. All patients were initially examined in our hospital with high suspicion of nasopharyngeal carcinoma, which was consistent with the clinical manifestations of nasopharyngeal carcinoma, and diagnosed with nasopharyngeal carcinoma after pathological biopsy. After diagnosis, either a single chemotherapy or 3-MA combined with chemotherapy was performed in our hospital. The age of patients ranged from 30 to 60 years. The patients were willing to cooperate with the medical staff in our hospital. A total of 375 subjects with complete cases were included in the study. The exclusion criteria included patients with cardiovascular and cerebrovascular diseases; patients with severe heart and lung function disorders; patients with other cancers; pregnancy; patients with chemotherapy tolerance; physically disabled patients; bed-ridden patients; patients with mental illness; and transferred patient. In total, 249 patients fulfilled the criteria and these included 152 males and 97 females, with an average age of 42.33 ± 12.83 years. Of these 249 subjects, 115 were treated with 3-MA chemotherapy (research group), while 134 subjects were treated with a single chemotherapy (control group).

Methods

All patients were treated with docetaxel and cisplatin (TP) chemotherapy in our hospital at a daily dose of 75 and 80 mg/m^2, respectively, as per the 2010 guidelines for radiochemotherapy [11]. Docetaxel was repeated every 3 weeks, and anti-allergic drugs (diphenhydramine [40 mg i.m.], cimetidine [0.4 mg i.v.], and dexamethasone [5 mg i.v.]) were administered 30 min before chemotherapy. The tumor target area was subjected to 70 Gy (2.0 Gy/cycle), and the clinical target area was given 54 Gy (20 Gy/cycle). The research group was treated with 30 mg of 3-MA (purchased from Sigma company in the United States) dissolved in 1 mL of water to obtain 200 mmol/L solution that was stored at 30°C and injected intravenously before each chemotherapy. The cancer tissue (2 mm) was obtained before chemotherapy as well as 15 and 30 days after chemotherapy.

Western blot analysis was performed to detect the expression of EGFR and VEGF proteins in the tissues. Proteins were extracted from cells using whole protein extraction kit and protein concentrations were determined by the bicinchoninic acid (BCA) protein quantitation kit. After discontinuous electrophoresis, the protein bands were transferred onto polyvinylidene fluoride (PVDF) membranes and the membranes were incubated in 5% non-fat milk for 20 h (4°C). After incubation, the membranes were washed thrice with phosphate buffer containing Tween (TPBS) and incubated at room temperature for 2 h. Images were obtained using the Bio-Rad gel imaging system to analyze the molecular weight and net optical density of the target bands.

The prognosis of the patients was followed up through phone, email, physical reexamination,

Table 1. Comparison of clinical information between two groups of patients [n (%)]

	Research group (n = 115)	Control group (n = 134)	X^2 or t	P
Age	43.27 ± 11.84	41.69 ± 12.54	1.017	0.310
Body weight (KG)	74.82 ± 15.33	76.92 ± 14.27	1.119	0.264
Disease course (d)	27.84 ± 16.53	28.42 ± 16.88	0.273	0.785
Gender			0.220	0.639
Male	72 (62.91)	80 (59.70)		
Female	43 (37.39)	54 (40.30)		
Place of residence			0.385	0.535
Town	89 (77.39)	108 (80.60)		
Rural	26 (22.61)	26 (19.40)		
Pathological stage			0.187	0.664
I~II	46 (40.00)	50 (37.31)		
III~IV	69 (60.00)	84 (62.69)		
Smoking			0.277	0.597
Yes	95 (82.61)	114 (85.07)		
No	20 (17.39)	20 (14.93)		
Pathological type			0.132	0.717
PDPC*	67 (58.26)	75 (55.97)		
UC#	48 (41.74)	59 (44.03)		

Note: * is poorly differentiated phosphorus cancer (PDPC); # is undifferentiated carcinoma (UC).

and home visiting for 5 years. The termination time and termination event were May 1, 2018.

Observation indicators

Clinical information (age, gender, weight, pathological stage, etc.) of the two groups of patients was obtained. Treatment effectiveness was divided into excellent treatment (complete tumor remission), effective treatment (partial remission of the tumor), ineffective treatment (no significant change in the tumor), and worsening treatment (no change in the tumor or even worsening of the disease) as per the 2010 Cancer Rehabilitation Guideline [12]. Effectiveness of treatment was calculated as follows: Effectiveness of treatment = (excellent treatment + effective treatment)/total number of cases × 100%. As per the 2010 CTCAE [13], treatment effectiveness was divided into grade I (mild adverse reactions such as nausea and vomiting), grade II (severe nausea, vomiting, and inflammatory reactions), grade III (including all adverse reactions mentioned above and arrhythmia), and grade IV (including all the above mentioned adverse reactions, visible bone marrow suppression, and organ toxicity situation). The incidence of adverse reactions was calculated as follows: Incidence of adverse reaction = (level III + IV)/total number of cases × 100%. Prognosis included patients with tumor metastasis; survival rates at 1, 3, and 5 years; and expression of EGFR and VEGF proteins during treatment.

Statistical method

SPSS22.0 statistical software was used to analyze and process the data. Count data such as patient's gender, pathological stage, and treatment efficiency were all expressed in the form of rate. Chi-square test was used for the comparison between groups. Measurement data such as patient age, weight, and EGFR protein expression level were expressed as mean ± standard deviation and *t*-tests were used for the comparison between the two groups. The survival rate was calculated using the Kaplan-Meier method and the survival rate was compared using the Log-rank test. A value of $P < 0.05$ was considered statistically significant.

Results

General data comparison

Comparison of the clinical conditions between two groups, including age, gender, weight, course of disease, family residence, pathological stage, pathological type, and smoking status, revealed no significant difference ($P > 0.050$), indicating that the results were comparable between the two groups of patients (**Table 1**).

Table 2. Comparison of treatment efficiency between two groups of patients [n (%)]

	Research group (n = 115)	Control group (n = 134)	X^2	P
Excellent treatment	62 (53.91)	45 (33.58)		
Effective treatment	42 (36.52)	51 (38.06)		
Invalid treatment	9 (7.83)	29 (21.64)		
Deteriorating treatment	2 (1.74)	9 (6.72)		
Treatment efficiency (%)	90.43	71.64	11.854	0.006

Table 3. The incidence of adverse reactions in the two groups of patients [n (%)]

	Research group (n = 115)	Control group (n = 134)	X^2	P
Class I	67 (58.26)	42 (31.34)		
Class I	33 (28.70)	44 (32.84)		
Class I	10 (8.70)	30 (22.39)		
Class I	5 (4.35)	18 (13.43)		
Incidence of adverse reactions (%)	13.01	35.82	16.992	< 0.001

Table 4. Comparison of tumor metastasis between two groups [n (%)]

	Research group (n = 115)	Control group (n = 134)	X^2	P
Tumor metastasis	8 (6.96)	24 (17.91)	6.630	0.010
No transfer	107 (93.04)	110 (82.09)		

Comparison of therapeutic efficiency

The therapeutic efficiency was 90.43% for the study group and was significantly higher (P = 0.006) than that reported for the control group (71.64%). Most of the patients from the study group underwent excellent treatment (53.91%), while those form the control group showed effective treatment (38.06%). In the study group, only 1.74% were categorized under worsening treatment, while 6.72% of patients from the control group were categorized under worsening treatment (**Table 2**).

Comparison of the incidence of adverse reactions

The incidence of adverse reactions was 13.04% in the study group and 35.82% in the control group. The difference was statistically significant (P < 0.001). Grade I adverse reactions were 58.26% in the study group but 32.84% in the control group. On the other hand, only 4.35% of patients from the study group exhibited grade IV adverse reactions, while 13.43% patients in the control group showed adverse reactions (**Table 3**).

Comparison of tumor metastasis

Of the 114 patients in the study group, only 6.96% of patients had tumor metastasis, while 17.91% patients from the control group exhibited tumor metastasis. The difference between the two groups was statistically significant (P = 0.010) (**Table 4**).

Comparison of EGFR and VEGF expressions

No significant difference in the expression levels of EGFR and VEGF was observed between the study and control groups before chemotherapy (P > 0.05). After 15 days of chemotherapy, EGFR and VEGF expressions were 525.74 ± 104.09 and 286.43 ± 45.66, respectively, in the study group and these values were significantly lower (P < 0.01) than those reported in the control group (618.08 ± 123.66 for EGFR and 318.42 ± 54.28 for VEGF). After 30 days of chemotherapy, EGFR and VEGF levels of 229.86 ± 52.53 and 127.33 ± 26.53, respectively, in the study group were significantly lower (P < 0.01) than those reported in the control group (387.45 ± 84.57 for EGFR and 228.69 ± 40.14 for VEGF). Changes in EGFR and VEGF expression levels were observed in the two groups, indicating that both groups showed a steady decline in the values with the time of chemotherapy. The decrease in the study group was more significant (**Tables 5** and **6**; **Figure 1**).

Comparison of prognosis

Of 249 patients, 234 patients were followed up with a success rate of 93.98%. Of the 15 patients lost to follow-up, 6 were in the study group and 9, in the control group. The 5-year overall survival rate was 71.56% for the study group and 54.07% for the control group. The

Table 5. Comparison of EGFR protein expression in tissues of two groups

	Research group (n = 115)	Control group (n = 134)	t	P
Before chemotherapy	864.24 ± 124.35	857.64 ± 134.92	0.399	0.690
Chemotherapy 15 d	525.74 ± 104.09*	618.08 ± 123.66*	6.313	< 0.001
Chemotherapy 30 d	229.86 ± 52.53*,#	387.45 ± 84.57*,#	17.324	< 0.001

Note: *represents the expression of EGFR protein compared with that before chemotherapy, P < 0.05; #represents the expression of EGFR protein compared with that 15 days after chemotherapy, P < 0.05.

Table 6. Comparison of VEGF Protein Expression in Tissues of Two Groups

	Research group (n = 115)	Control group (n = 134)	t	P
Before chemotherapy	427.34 ± 86.13	434.52 ± 90.24	0.639	0.523
Chemotherapy 15 d	286.43 ± 45.66*	318.42 ± 54.28*	4.988	< 0.001
Chemotherapy 30 d	127.33 ± 26.53*,#	228.69 ± 40.14*,#	23.092	< 0.001

Note: *represents the expression of VEGF protein compared with that before chemotherapy, P < 0.05; #represents the expression of VEGF protein compared with that 15 days after chemotherapy, P < 0.05.

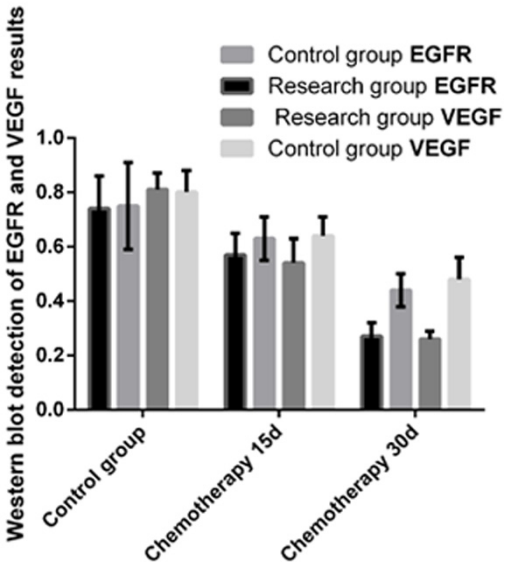

Figure 1. Western blot detection of EGFR and VEGF results. There was no significant difference in EGFR before chemotherapy between the two groups, which decreased after chemotherapy, and the decline was more significant in the study group. There was no significant difference in VEGF before chemotherapy between the two groups, which decreased after chemotherapy, and the decline was more significant in the study group.

difference in the survival rate between the two groups was statistically significant (P = 0.010) (**Figure 2**).

Discussion

Many studies [14, 15] have highlighted the decreased sensitivity of nasopharyngeal cancer cells to chemotherapy as the main cause of poor prognosis of nasopharyngeal carcinoma after chemotherapy. Chemotherapy is associated with major side-effects. The chemotherapeutic drug cisplatin is an inorganic metal complex that gets hydrolyzed and crosslinked with the DNA of tumor cells after entering cancer cells, resulting in the inhibition of DNA replication [16]. In addition, the radiation effect of chemotherapy causes a series of damages, including destruction and shedding of DNA bases and DNA strand break, that serve as the key factors leading to the changes in the patients' cellular biological functions [17]. The stress-autophagy reaction of the endoplasmic reticulum has gathered considerable attention in oncology studies. Autophagy can not only protect cells but also supplement the metabolic function of organelles and biological activities. 3-MA has a strong regulatory effect on cell proliferation and apoptosis and may counteract chemotherapeutic sensitivity caused by cisplatin and improve the efficiency of chemotherapy [18]. However, the lack of research on 3-MA in nasopharyngeal carcinoma has restricted its application for the treatment of nasopharyngeal carcinoma. Therefore, this study serves as a reference for clinical practice by comparing the therapeutic differences between 3-MA combined with chemotherapy regimen and traditional single chemotherapy regimen in nasopharyngeal carcinoma and detecting the expression levels of VEGF and EGFR in patients undergoing chemotherapy.

The results of this experiment showed that the treatment group using 3-MA combined with TP chemotherapy was significantly better than the

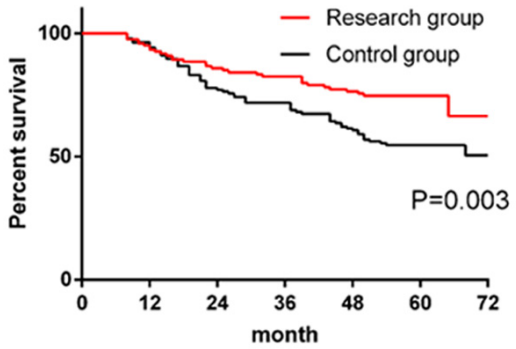

Figure 2. Prognostic survival curves for both groups. The survival rates of patients in the study group at the 1st, 3rd, and 5th year were 92.66%, 77.06%, and 71.56%, respectively. The survival rates at the 1st, 3rd, and 5th year in the control group were 94.07%, 66.67%, and 54.07%, respectively. The 5-year survival rate was significantly higher in the study group than in the control group (P = 0.003).

control group patients who used single TP chemotherapy in treatment efficiency, adverse reaction, tumor metastasis and prognosis, which are consistent with study of Pickard et al. on the chemotherapy of 3-MA in prostate cancer [19]. In the course of chemotherapy, the expression of VEGF and EGFR in the study group gradually decreased, but the decline in the study group was more significant, indicating that the effect of treatment group was better. The reason is presumed that in nasopharyngeal carcinoma, abnormal activation and overexpression of VEGF and EGFR can not only transform normal cells in the patient's body into tumor cells, but also promote tumor metastasis and invasion through the autocrine loop [20]. During the course of chemotherapy, the patient's endoplasmic reticulum undergoes a stress response due to the stimulation of chemotherapy and cisplatin, and the trophic factors required for normal metabolism are largely lost [21]. At this time, supplementation of 3-MA enhances the survival of cytoplasmic macromolecules and organelles, and the cytokine's ability to degrade in double-layered vesicles is reduced [22], so that its contents can continue to participate in the normal metabolism of the endoplasmic reticulum, which greatly enhances the chemosensitivity of patients during chemotherapy. The study by Zhang et al. [23] pointed out that VEGF and EGFR accelerate the proliferation and division of tumor cells and promotes the formation of tumor blood vessels by phosphoinositide 3-kinase (P13K)/protein kinase B (AKT) signaling pathway. While 3-MA is an inhibitor of P13K activity [24]. Thus, the ability of VEGF and EGFR to promote tumor proliferation and metastasis is inhibited as well, which results in the difference of VEGF and EGFR expression between the two groups. The angiogenic capacity of VEGF and EGFR is also responsible for tumor invasion and metastasis [25]. Inhibition of their expressions not only reduce the toxic side-effects of chemotherapy drugs but also decreases the risk of tumor metastasis [26]. These effects cause differences in the prognosis of tumor metastasis and survival rates between the two groups, suggesting that 3-MA increases the chemotherapy sensitivity in the treatment of nasopharyngeal carcinoma. A similar study also found that 3-MA can sensitize HONE-1 cells to chemotherapy and radiotherapy, which is related to prevention of endoplasmic reticulum stress-induced autophagy in nasopharyngeal carcinoma cells [27]. But the mechanism behind it requires more detailed research.

Our study has some limitations such as the small base of the research object and the relatively single population. The effect of 3-MA on the sensitivity of chemotherapy and VEGF as well as EGFR expressions in the tissues of patients with nasopharyngeal carcinoma needs to be further analyzed. We will conduct a longer-term follow-up survey of the subjects of this study and continue to study the detailed mechanism of action of 3-MA to achieve best therapeutic results.

In summary, 3-MA combined with TP chemotherapy regimen is significantly superior to single TP chemotherapy for the treatment of nasopharyngeal carcinoma and may inhibit the expression of VEGF and EGFR in patient's tissues.

Disclosure of conflict of interest

None.

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