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Effects of docetaxel plus three-dimensional conformal radiation therapy on microvessel density and apoptosis expression in local advanced squamous non-small-cell lung cancer

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ABSTRACT. We examined the effects of weekly single-agent docetaxel plus three-dimensional conformal radiation therapy (3D-CRT) on apoptotic index (AI) and microvessel density (MVD) in local advanced non-small-cell lung squamous cancer patients and analyzed the correlation of MVD, AI, and 50% tumor shrinkage time (T0.5) The molecular mechanism of docetaxel radiosensitization was investigated. Sixty untreated patients with stage IIIA or IIIB lung squamous cancer were enrolled and randomly divided into two groups: observation (N = 30; 3D-CRT + docetaxel + adjuvant chemotherapy) and control (N = 30; 3D-CRT + adjuvant chemotherapy). From day 1 radiotherapy, the observation group received intravenous docetaxel (36 mg/m^2) once weekly for 6 weeks. Post-radiotherapy, chemotherapy of docetaxel combined with cisplatin lasted 4-6 cycles in both groups. Before radiotherapy and within 24 h after radiotherapy (20 Gy), bronchoscopic biopsy was performed twice at the same site. To analyze the MVD of tumor specimens with immunohistochemical staining . The AI of

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lung cancer cells was assessed with TUNEL assay, T0.5 values were calculated. The observation group had significantly lower MVD than the control group ($P < 0.05$). AI significantly increased before and after treatment in the observation group compared with the control group ($P < 0.05$). The decreased MVD values negatively correlated with T0.5 values ($r = -0.624$, $P < 0.05$), whereas the increased AI values did not correlate with the T0.5 values. Docetaxel radiosensitization may occur by decrease in MVD and increase in AI values. Weekly single-agent docetaxel plus 3D-CRT can improve prognosis and quality of life in local advanced non-small-cell lung squamous cancer patients.

Key words: Docetaxel; Squamous cell carcinoma; Radiotherapy; Microvessel density; Apoptosis

INTRODUCTION

Local advanced non-small-cell lung cancer cases were accounts for approximately 40% of the diagnosed patients. Concurrent chemoradiotherapy is the standard treatment. However, there were no standard regimen of chemotherapy and radiotherapy doses. We examined the therapeutic effects of single-agent docetaxel plus three-dimensional conformal radiation therapy (3D-CRT) in patients with local advanced non-small-cell lung cancer, which had been shown a good efficacy and the results can be seen as follows.

MATERIAL AND METHODS

General information

The inclusion criteria were as follows: patients with histologically diagnosed squamous cell carcinoma; age ≤ 76 years; Karnofsky score ≥ 70 points; no serious internal diseases that may affect treatment completion (chronic obstructive pulmonary disease and pulmonary heart disease; patients with severe infection were enrolled after the infection was controlled or reduced); no history of chemotherapy and chest radiation therapy; presence of measurable or evaluable lesions. The patients and their families signed the consent forms. The expected survival time was >6 months. Normal values of routine blood test parameters were described as follows: white blood cell count ≥4.0 x 10^9/L; platelet count ≥75 x 10^9/L; red blood cell count ≥4 x 10^9/L. Normal hepatic function was characterized as follows: bilirubin ≤2.0 mg/dL; albumin ≥2.5 g/dL; aspartate aminotransferase and alanine aminotransferase ≤3-5X upper normal limit (normal range, 5-64 IU/L); prothrombin time/international normalized ratio ≤1.5; normal renal function.

The exclusion criteria were as follows: pregnant or breastfeeding women; significant organ failure such as heart, liver, kidney, or lung failure; organ metastasis; patients allergic to alcohol or other biological agents.

Patient grouping

Between April and November 2008, 60 untreated patients with stage IIIA or IIIB local

advanced squamous cell lung cancer were enrolled in this study performed at the cancer center of our hospital. Patients were aged between 18 and 76 years; the median age was 57 years. The patients were randomly divided into two groups: the observation group (30 patients; 3D-CRT plus docetaxel + adjuvant chemotherapy) and the control group (30 patients; 3D-CRT + adjuvant chemotherapy). From day 1 of radiotherapy, the observation group received intravenous docetaxel (36 mg/m^2) once weekly for 6 weeks. After radiotherapy, adjuvant chemotherapy lasted 4 cycles and 4-6 cycles in the observation and control groups, respectively. Recombinant human granulocyte colony-stimulating factor was administered when patients developed neutropenia during the treatment period. Both groups had the same pathological type of tumor, and there was no significant difference in the clinical staging between the groups.

Methods

A linear accelerator (precise type, Sweden) and the following reagents were used: 1:30 mouse anti-human CD31 monoclonal antibody (Beijing Zhongshan Company); peroxidase-labeled streptomycin avidin (streptavidin/peroxidase); a staining kit (Beijing Zhongshan Company); and an *in situ* cell death detection kit (Roche Inc.).

Tissue collection was performed as follows: before radiotherapy and 24 h after radiotherapy (20 Gy), bronchoscopic biopsy (Fujinon EB-270S) of the lung lesions was performed, and at both times, tumor specimens were obtained from the same site (by the same physician; the location was radiographed and marked).

Microvessel density (MVD)

Immunohistochemical staining (streptavidin-peroxidase method) of tumor specimens was performed according to the kit manufacturer instructions. According to Weidner method, the fields with clear stained vascular endothelial cells of the cancerous tissue and good background control and highest capillaries density were collected. The number of blood vessels of five fields (400X) was counted. The area was 0.43 mm^2 with each 400 times view field. The number of capillaries was calculated per mm^2, MVD was calculated. Decrease in MVD (root/mm^2) = MVD (before radiotherapy) - MVD (after radiotherapy).

In situ apoptotic index (AI)

The terminal deoxynucleotidyl transferase dUTP nick end labeling method was used for assessing the AI of the lung cancer cells. Positive expression of apoptotic cells (including apoptotic bodies) was defined as the appearance of brown particles in the cell nuclei. Fields with evenly distributed cells were selected under low magnification (100X), and the number of positive cells per 1000 tumor cells was counted under high magnification (400X). Ten fields were repeatedly examined, and the percentage of positive cells in the fields was calculated as the AI. Radiation-induced AI (%) = AI (after 20-Gy radiotherapy) - AI (with no radiation).

Time for 50% local shrinkage of lung tumors (T0.5)

Tumor size was measured once every week by using chest computed tomography (CT). For the second time, the thin-section CT scans were performed only on the mass (the site was

determined by the 3D-CRT treatment center). T0.5 in lungs was calculated.

Radiotherapy techniques

Radiotherapy was performed using a 6-MV linear accelerator, and 3D-CRT and a conventional split dose regimen were used for *in vitro* irradiation. Patients took natural posture, the body molding shape was made for fixation, laser spots were marked. Spiral CT with thin slices (3 mm) was performed, and the CT data were sent to treatment planning system workstations. Two radiation oncologists and one radiologist together performed target delineation. Target volume was defined according to the guidelines of the International Commission on Radiation Units and Measurements, Report No. 50. Gross tumor volume included primary lesions and enlarged mediastinal lymph nodes with a short-axis diameter >1 cm. Clinical target volume (CTV) of lower and middle lobe lung included the ipsilateral hilum, subcarinal nodes, aortopulmonary window, pretracheal retrocaval lymphatic drainage area. Radiotherapy was provided when positive lymph nodes (>1 cm) were present in the upper mediastinal lymph drainage area, whereas it was not provided when lymph nodes were negative. CTV in upper lobe lung cancer included the ipsilateral hilar and subcarinal nodes, primary pulmonary window, and lymphatic drainage area behind the tracheal vena cava. Radiotherapy was provided when positive subcarinal lymph nodes were present, whereas it was not provided when lymph nodes were negative. Breathing mobility, mechanical and setup errors, and other factors were considered when calculating the planning target volume (PTV). The treatment plan was optimized using dose-volume histogram, and PTV was covered by 90% of isodose curves (V20 values ≤25% and mean dose ≤30 Gy for both lungs; maximum dose of spinal irradiation ≤45 Gy, each spinal dose ≤2 Gy; esophagus Dmax ≤80 Gy, V50 values ≤20%; heart V65 values ≤33%, V50 values ≤65%). A 3D-CRT planning system was used for the result calculation. The radiotherapy position and reference points were set on the CT simulator, and the accuracy of the radiation fields was verified. The plan irradiation dose was DT60-68Gy, the preventive dose of lymphatic drainage area was DT45-50Gy, DT1.8-2.0 Gy/time, 1 time/day, 5 days/week.

The evaluation indicators were MVD, AI, and T0.5. The conditions for treatment termination were severe allergic reactions, severe liver and kidney dysfunction, voluntary termination of therapy, and development of grade IV myelosuppression.

Follow-up

After treatment, follow-up was performed every 3 months in the first year and every 6 months in second year. We estimated the recurrence or metastasis by imaging data including ultrasonography, CT, magnetic resonance imaging (MRI), and positron emission tomography (PET)-CT. If there were symptoms or signs occurred in the patients, the recurrence or metastasis was determined based on image data by performing ultrasound, CT, MRI, and PET-CT. All patients were followed up for at least 3 years until December 2011. Three patients were lost to follow-up including the death cases. The follow-up rate was 95.6%.

Statistical analysis

The SPSS 10.0 software package was used for statistical analysis. Survival and local control rates were analyzed using the Kaplan-Meier method. Decreased MVD, treatment-induced

AI, and T0.5 values between the two groups were compared using the Mann-Whitney *U*-test. The correlation between T0.5, decreased MVD, and treatment-induced AI values was analyzed using the Spearman correlation test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Decrease in MVD

During DT 20-Gy radiotherapy, MVD decreased to varying degrees between the two groups, they were 16.00 root/mm^2, 2.00 root/mm^2 in the observation group and control group, $P < 0.01$. The difference was statistically significant (Table 1, Figure 1A and B).

Table 1. Differences in MVD, AI, and T0.5 before and after treatment between the two groups (median number).

Groups	Cases	MVD (vessels/mm^2)			AI (%)			T0.5 (days)
		Before treatment	After treatment	Decrease in expression	Before treatment	After treatment	Increase in expression	
Observation group	30	60.00 (12-136)	49.00 (7-120)	16.00# (3-43)	0.31 (0.20-0.50)	0.84 (0.40-1.09)	0.54# (0.16-0.73)	15.0# (5.0-20.0)
Control group	30	62.00 (25-148)	55.00 (16-120)	2.00 (0.12-12)	0.35 (0.18-0.42)	0.53 (0.46-1.13)	0.24 (0.01-0.56)	25.0 (8.0-28.0)
p				<0.01			<0.05	<0.05
r								-0.624

#Compared to the control group, the difference is statistically significant ($P < 0.05$).

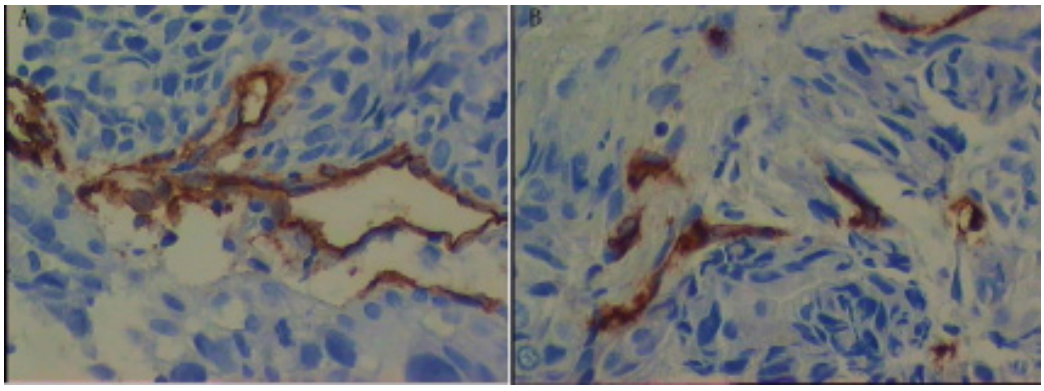

Figure 1. Differences in microvessel density (MVD) between the two groups after treatment. **A.** Streptavidin-peroxidase (SP), 400X; control group. **B.** SP, 400X; observation group. MVD was significantly lower in the observation group than in the control group ($P < 0.05$).

Changes in AI

During DT 20-Gy radiotherapy, AI increased to varying degrees and was observed to be significantly higher in the observation group than in the control group (0.54 *vs* 0.24) (Table 1, Figure 2A and B).

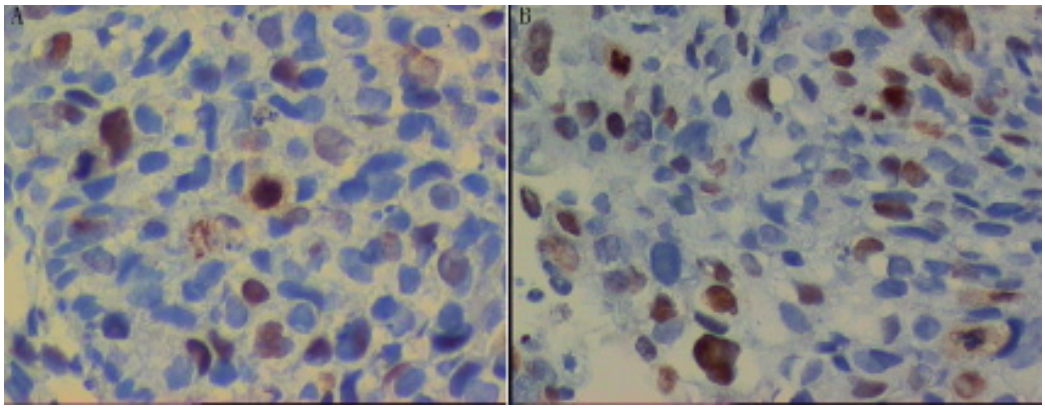

Figure 2. Differences in apoptotic index (AI) between the two groups after treatment. **A.** SP, 400X; control group. **B.** SP, 400X; observation group. AI was significantly higher in the observation group than in the control group ($P < 0.05$).

Changes in T0.5

The T0.5 values in the observation and control groups were 15 and 25 days, respectively ($P < 0.05$). T0.5 was shorter in the observation group (concurrent docetaxel and 3D-CRT) than in the control group (Table 1).

The decreased MVD values negatively correlated with T0.5 ($r = -0.624$; $P < 0.05$). The more significant the decrease in MVD, the faster the tumor regression (Table 1).

DISCUSSION

The 5-year survival rate of patients undergoing conventional radiation therapy for local advanced non-small-cell lung cancer is only 4-10%. Concurrent chemoradiotherapy is the standard treatment, but its efficacy is still not satisfactory. Local recurrence and distant metastasis remain the main causes of failure (Vokes et al., 2007). How to improve the local control rate and reduce the rate of distant metastasis become the focus of cancer researches. An insufficient radiation dose is closely related to local failure, and increasing the radiation dose within a certain range may improve the local control rate (Wu et al., 2007). However, owing to the potential risk of organ damage, the radiation dose cannot be increased to the desired level. Therefore, because radioactive toxicity and complications rise with an increase in the radiation dose for improving the local control rate, this method has rarely been adopted in clinical practice. 3D-CRT increases the target dose to some extent, and to better protect the organs at risk, it reduces radioactive toxicity to the maximum degree, thereby improving the quality of life in patients.

The emergence of new chemotherapy drugs has led to the improvement in the efficacy of treatment for local advanced squamous non-small-cell lung cancer. Docetaxel is one of the most effective single-agent drugs, and phase II clinical trials have shown that relatively low doses of docetaxel (36 mg/m^2 weekly) in the treatment of advanced non-small-cell lung cancer can significantly reduce bone marrow suppression and nonhematologic toxicity. In one study, docetaxel efficiency was 20-26%, and the 1- and 2-year survival rates of patients were 28 and 15%, respectively (Hainsworth et al., 2001). A Canadian study showed that 75

mg/m^2 docetaxel, compared with the best supportive treatment, prolonged the survival rate of patients, and the side effects of this treatment could be tolerated (Sun et al., 1998; Hainsworth et al., 2001; Dancey et al., 2004). Several studies (Zheng et al., 2006; Mo et al., 2008) have reported data on concurrent docetaxel and radiotherapy for phase III non-small-cell lung cancer patients; however, no standard regimen of chemotherapy and radiotherapy doses has yet been established. Therefore, we examined the effects of weekly single-agent docetaxel plus 3D-CRT on AI and MVD in patients with local advanced squamous non-small-cell lung cancer.

In 1990, Folkman (1990) proposed a hypothesis that tumor growth depended on angiogenesis. MVD was associated with tumor stage, invasion, metastasis, and prognosis (Liu et al., 2005). Tumor angiogenesis was necessary for maintaining tumor metastasis and growth. The number of tumor microvessels was considered as an important indicator of prognosis and efficacy assessment of radiation therapy in patients with solid tumors; apoptosis was an indicator for radiation sensitivity in lung cancer. Previous studies (Fischer et al., 2001; Hennequin, 2004) have reported that docetaxel may have radiosensitization effects such as cell cycle reoxygenation, radiation-induced apoptosis, and other synergistic antiangiogenic effects. In the present study, MVD (median) in the observation group decreased from 60.00 to 49.00, and MVD expression decreased to 16.00; MVD (median) in the control group decreased from 62.00 to 55.00, and MVD expression decreased to 2.00. In the observation group, the decrease in MVD was more obvious ($P < 0.05$), suggesting that concurrent docetaxel and 3D-CRT reduced tumor angiogenesis. AI in the observation group increased by 0.54, whereas that in the control group increased by 0.24. Increase in the AI values in the observation group was more significant ($P < 0.05$), suggesting that concurrent docetaxel and 3D-CRT improved the sensitivity of radiotherapy. Median T0.5 was 15 days in the observation group and 25 days in the control group; local T0.5 in the observation group was shorter, and local tumor regression was faster ($P < 0.05$). T0.5 and decreased MVD negatively correlated with each other ($r = -0.624$), which indicates less angiogenesis and more significant tumor regression. No correlation was observed between the increased AI values and T0.5; this shows that docetaxel may produce sensitizing effects via antiangiogenesis and apoptosis induction.

Zhou et al. (2003) reported the results of clinical randomized controlled trials on the surgical treatment of phase III non-small-cell lung cancer after neoadjuvant chemotherapy at the 10th International Conference on Lung Cancer, Vancouver. In Group A patients, the 1-, 3-, 5-, and 10-year survival rates were 89.35, 67.46, 34.39, and 29.34%, respectively. In our study, the 1- and 3-year survival rates were similar to those reported by Zhai (2012). Radiochemical resynchronization therapy aggravated acute radiation injury in phase I-II patients; however, the patients can tolerate these adverse effects after symptomatic treatment.

In summary, chemoradiotherapy may be satisfactory with weekly docetaxel plus 3D-CRT in patients with local advanced squamous non-small-cell lung cancer, in the presence of strict indications and absence of contraindications with positive symptomatic and supportive treatment. Docetaxel may cause antiangiogenic effects, suppress the generation of microvessels, induce tumor cell apoptosis to produce radiosensitization effects and accelerate tumor regression, and improve the local control and long-term survival rates. T0.5 and MVD negatively correlated with each other, but no correlation was observed between the increased AI values and T0.5. This study has presented a rational antitumor treatment option and provides valuable information to clinicians.

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