

## **Relationships between CD44, hyaluronic acid expression and lymphatic metastasis and radiosensitivity of nasopharyngeal carcinoma.**

**De Yu Sun\***, Hong Yu, Xue Bin Qiu, Guang Li, Na Zhang

Cancer Hospital of China Medical University, Liaoning Cancer Hospital & Institute, Shengyang 110042, Liaoning Province, PR. China

### **Abstract**

To study the sensible relationships between CD44, Hyaluronic acid (HA) expression and lymphatic metastasis and radiosensitivity of nasopharyngeal carcinoma and to explore and predict effective biological indicators for potential lymphatic metastasis and radiosensitivity. Immunohistochemistry and RT-PCR method have respectively tested the CD44 protein, levels of HA and mRNA expression in 50 patients with nasopharyngeal carcinoma. CD44 protein has expressed in either nasopharyngeal carcinoma's cell membrane or cytoplasm. The positive rate of protein expression in N(+) group was 72.0% and 36.0% for N(-) group. There was a statistical difference between two groups,  $P=0.027$ . Three months after radiotherapy, the positive rate was 61.1% in the metastatic lymph node fading group and 100% in the completely fading group. It had a difference,  $P<0.05$ . RT-PCR result showed that CD44 mRNA expression in the N(+) group was higher than that of N(-) group. Relative contents were  $1.0584 \pm 0.3958$  and  $0.4738 \pm 0.3462$ , respectively. It had a statistical difference,  $P=0.000$ . The relative contents among incomplete radiotherapy fading group and complete radiotherapy fading group were  $1.4282 \pm 0.3116$  and  $0.9146 \pm 0.3298$ , respectively in N(+) group. It had a statistical difference,  $P=0.002$ . The relative contents in cornification group and non-cornification group were  $0.8365 \pm 0.4706$  and  $0.5118 \pm 0.3627$ , respectively. It also had a statistical difference,  $P=0.048$ . Cancer tissue's HA and mRNA (RT-PCR) expression levels in N(+) group were higher than that of N(0) group ( $P<0.05$ ). Cervical lymph nodes in the incomplete fading group were significantly more than that of complete fading group (all  $P<0.05$ ). The results showed that HA and CD44mRNA expression in nasopharyngeal carcinoma had significant differences between lymph node metastasis group or non-metastasis group, lymph node fading group or non-fading group. The relative contents of HA, CD44 mRNA and CD44 protein in the lymph node metastasis group were significantly higher than that of non-lymph node metastasis group. The relative contents of HA, CD44 mRNA and CD44 protein in the postradiotherapy incomplete lymph node fading group were significantly higher that of postradiotherapy complete lymph node fading group.

**Keywords:** Nasopharyngeal carcinoma, lymph node metastasis, radiotherapy, CD44.

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### **Introduction**

Tumor invasion or metastasis is a complex process. Nasopharyngeal carcinoma has characteristics of early metastasis and high grade malignancy, mainly of lymph node metastasis. It has a higher cervical lymph node metastasis as the clinical manifestation. CD44 (the No.44 of differentiation), which was a kind of transmembrane glycoprotein, has been found and confirmed in 1980. As a transmembrane glycoprotein, CD44 is generally existed in human cells and plays an important role in cell-cell, cell-matrix interaction, takes part in multiple functions such as cell adhesion, lymphocyte homing and cell's internal or external signaling. CD44, recently has been found closely associated with human tumors, especially in growth and metastasis of malignant tumors. Hyaluronic acid (HA) is a macromolecule glucosamine glycan. Studies have revealed that HA is composite by

interstitial cell's plasma membrane and released [1] as a soluble product and then enters into the blood stream via lymph circulation thereby participating the formation of proteoglycan which is a main ingredient [2] of matrix in the connective tissue. HA plays an important role in interstitial metastasis, tumorous invasion, differentiation of foetal deformity cancer cells, formation of embryonal tissue and viral conversion process in the fibrocytes via acting on cells and ICM receptors. It has been studied clearly so far, HA receptors has been divided into four major categories-CD44 (cluster of differentiation CD44), RHAMM (receptor for HA-mediated motility), IV d4 and LEC (Liver Endothelial Call Receptor). HA, which has effects in promoting adhesion and metastasis, plays an important role in tumor growth and progression. Extracellular matrix of hyaluronic acid provides molecular basis for biological effects such as cell message passing, cell

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morphology control, movements and proliferation and has vital functions [3] in tumor growth, tumor angiogenesis and metastasis. HA effect in current anti-tumor studied is achieved by combination of HA and CD44. At present, the specific mechanism of signal transduction pathway of HA and CD44 in regulation of tumorous lymphatic metastasis has remained unknown. Studies of nasopharyngeal carcinoma-related signal pathway have yet reported at home and abroad. Therefore, we applied the immunohistochemistry and RT-PCR method to detect HA and CD44 expression patterns in a level of protein and mRNA for discussion of relationships between HA, CD44 expression and lymphatic metastasis and radiosensitivity of nasopharyngeal carcinoma, which will provide biological indicators in prediction of potential lymphatic metastasis and radiosensitivity in nasopharyngeal carcinoma.

**Materials and Methods**

**Sample source**

A total of 50 patients with nasopharyngeal carcinoma treated at the Department of Radiotherapy between June 2009 and December 2013 in Liaoning Cancer Hospital & Institute have not involved in other treatments before radiotherapy. Among them there were 26 male subjects and 21 female subjects aged between 12 and 75 years old, the mean age of 44.5 years old and the median age of 47 years old. There were 25 patients with lymph node metastasis [N(-)] and 25 patients without lymph node metastasis [N(+)] (enhanced MRI for confirmed diagnosis); 25 patients with T1-2 and 25 patients with T3-4 (Refer to 2008 Nasopharyngeal Carcinoma Staging Criteria); 10 patients with keratinizing carcinoma and 40 patients with non-keratinizing carcinoma. Specific T and N Classification for nasopharyngeal carcinoma is shown in Table 1.

**Table 1.** Specific classification (cases) for nasopharyneal carcinoma.

N	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>4</sub>	Total
N(-)	6	8	9	2	25
N(+)	5	6	13	1	25
Total	11	14	22	3	50

**RT-PCR samples:** Nasopharyngeal carcinoma tissue blocks were taken respectively by a plier within 30 minutes after in vitro and placed in the quick-freezing tubes with liquid nitrogen for quick freezing and then transferred into a refrigerator under 80 for storage.

**Immunohistochemical samples:** Nasopharyngeal carcinoma tissue blocks were taken by a plier adjacent to RT-PCR sample margin and fixed by 10% of neutral formalin, embedded by paraffin, and made to serial sections with 4cm in thickness. All the patients in this study have signed the informed consent form, and this stidy was approved by the Ethics Committee of

Liaoning Cancer Hospital & Institute, Dalian Medical University Clinical Oncology College.

**Table 2.** Nasopharynx cancer CD44 protein expression.

Group	N	+++	++	+	-	Positive rate	expression
N(+)	25	1	12	5	7	0.72	
N(0)	25	0	5	4	16	0.36	

Statistical analysis result:  $\chi^2=7.188$ ,  $P=0.027$ , two groups has significant difference ( $P<0.05$ ).

**Main reagents**

**RT-PCR reagents:** Total RNA extraction kit is purchased from Henan Sino-American Biotechnology Co., Ltd, RT-PCR kit is purchased from TaKaRa (Dalian) Engineering Co., Ltd, DNA MARKER is purchased from Dalian Boride biotechnology Co., Ltd, and primer is compounded by from Shanghai Bioasia biotechnology Co., Ltd. Immunohistochemistry reagents: DAB kit and mouse anti-human monoclonal antibodies are purchased from Fujian Maxim Biological Engineering Co., Ltd.

**Table 3.** Nasopharynx cancer CD44 protein expression.

Group	N	+++	++	+	-	positive expression rate
N(+) radiotherapy not complete dissipating	7	1	6	0	0	1
N(+) radiotherapy dissipating	18	0	6	5	7	0.611

Statistical analysis result:  $\chi^2=8.974$ ,  $P=0.011$ , two groups has significant difference ( $P<0.05$ ).

**Table 4.** Nasopharynx cancer CD44 mRNA expression results and the relative content ( $\bar{X} \pm S$ ).

Group	N	Relative content
N(+)	25	1.0584 ± 0.3958
N(-)	25	0.4738 ± 0.3462

Statistical analysis result:  $t=-5.557$ ;  $P=0.000$ , two groups has significant difference ( $P<0.05$ ).

**Experimental methods**

**Immunohistochemical staining:** conventional Streptomyces antibiotic proteins -streptvidin-peroxidase (Streptavidin peroxidase, SP) immunohistochemical SP method with mouse anti-human CD44 that is, monoclonal antibody is used as an anti-detected cancerous tissue CD44 protein expression. CD44 protein positive expression is brown particles, mainly existing in the cell membrane and cytoplasm. Semi-quantitative scoring AB value method is adopted to calculate the results.

**RT-PCR method:** Total RNA extraction: extract total RNA according to kit requirement, after washed and dried with ethyl

alcohol, the RNA was dissolved with Rnase Free Water and stored for use at -20 . Test RNA concentration and purity with ultraviolet spectrophotometer. cDNA Synthesis reaction system: RNA 2  $\mu$ l, 2  $\times$  buffer 10  $\mu$ l, 25 mmol/L MgSO<sub>4</sub> 4  $\mu$ l, dNTPs (10 mmol/L) 1 $\mu$ l, AMV (22U/ $\mu$ l) 1  $\mu$ l, Oligo dT 15 (50 mmol/L) 1  $\mu$ l, RNase Inhibitor 0.5 $\mu$ l, RNase-Free H<sub>2</sub>O 0.5 $\mu$ l. Reaction condition: 65°C for 1 min; 30°C for 5 min; increase to 65°C constantly within 15 min-30 min; 98°C for 5 min; 5°C for 5 min. CD44 reaction system: cDNA 3 $\mu$ l, RNase-Free dH<sub>2</sub>O 17.1 $\mu$ l, 10  $\times$  buffer 2.5  $\mu$ l, dNTPs (2.5 mmol/L) 2  $\mu$ l, Taq E (5U/ $\mu$ l) 0.2  $\mu$ l, CD44-F 0.1  $\mu$ l, CD44-R 0.1  $\mu$ l. Reaction condition: 94°C for 3 min; 94°C for 40s $\rightarrow$ 51.0°C for 1 min $\rightarrow$ 72°C, repeating 35 circulations; 72°C extending for 7 min. CD44 (321 bp) upstream primer: 5'-TTGTG-GCATTATTCATCAG-3', and T3 in downstream primer: 5'-GGTAGACAGGGAGGAG-CA-3';  $\beta$ -actin (498 bp) upstream primer: 5'-GTGGGGCGCCCCAGGCAC-CA3', downstream primer: 5'-CTCCT-TAATGTCACGCACGATTTTC-3'. Analysis of the product: 2% agarose gel after electrophoresis, place it under ultraviolet light and observe CD44 and actin amplified band product, use 1D Kodak gel imaging analysis system to detect the amplified product content and calculate it with the following formula: relative content of CD44 gray value/ $\beta$ -actin gray value. Three months after the radiotherapy, enhance MRI check to determine whether the neck N (0) is completely dissipated.

### Statistical analysis

Perform analysis using SPSS12.0 statistical software, count data using the 2 test, measurement data using independent sample t test and paired t test, P<0.05 is considered statistically significant.

**Table 6.** CD44 protein and mRNA expression in Nasopharynx cancer and the relationship with clinicopathological parameters.

Group	N	CD44 protein positive rate (%)	Statistical analysis result		D44mRNA relative content ( $\bar{X} \pm S$ )	Statistical analysis result	
			$\chi^2$ value	P value		t value	P value
<b>Age</b>							
$\leq 61$	41	51.22	5.226	0.073	0.7293 $\pm$ 0.4667	-1.184	0.242
>61	9	66.67	-	-	0.9341 $\pm$ 0.4859	-	-
<b>Gender</b>							
Male	26	50	1.536	0.464	0.7859 $\pm$ 0.5076	0.305	0.762
Female	24	58.33	-	-	0.7448 $\pm$ 0.4397	-	-
<b>T grade</b>							
T1-2	25	52	0.155	0.926	0.7632 $\pm$ 0.4796	-0.044	0.965
T3-4	25	56	-	-	-	-	-
<b>Tumor differentiation</b>							
High and medium Differentiation	10	50	3.94	0.139	0.8365 $\pm$ 0.4706	2.030	0.048
Low differentiation	40	65.8	-	-	0.5118 $\pm$ 0.3627	-	-

## Results

### Nasopharynx cancer CD44 protein expression

CD44 cytoplasmic membrane proteins are expressed in cancer cell membrane and cytoplasm. With lymph node metastasis and without lymph node metastasis expression rate difference is significant (P<0.05), three months after radiotherapy, positive expression rate of lymph node metastasis dissipating and not complete dissipating group are significantly different (P<0.05). (See Tables 2, 3 for details).

### Nasopharynx cancer CD44 mRNA expression

Expression of cancer tissue of CD44 mRNA (RT-PCR) N(+ group is higher than that of N(0) group (P<0.05), three months after radiotherapy, not complete dissipating group of cervical lymph nodes is significantly higher than that of dissipating group (P<0.05). Relative content of CD44 expression in cancer tissues compared with internal reference bands are shown in Tables 4-6.

**Table 5.** Nasopharynx cancer CD44 mRNA expression results and the relative content ( $\bar{X} \pm S$ ).

Group	N	Relative content
Not complete dissipating group after radiotherapy	7	1.4282 $\pm$ 0.3116
Complete dissipating group after radiotherapy	18	0.9146 $\pm$ 0.3298

Statistical analysis result: t=-3.546, P=0.002, two groups has significant difference (P<0.05)

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In addition to tumor differentiation of CD44 mRNA in high medium differentiation and low differentiated between the two groups is significant (P<0.05), the remaining parameters are not significant.

**Table 7.** Nasopharynx cancer HA mRNA expression result and relative content ( $\bar{X} \pm S$ ).

Group	N	Relative content ( $\bar{X} \pm S$ )
N(+)	25	1.503 ± 0.378
N(-)	25	1.069 ± 0.374

Statistical analysis result: t value = 5.330; P Value=0.000,two groups has significant difference (P<0.05)

**Table 8.** Nasopharynx cancer HA mRNA expression result and relative content ( $\bar{X} \pm S$ ).

Group	N	Relative content
N(+) not complete dissipating group	7	1.4118 ± 0.2925
N(+) complete dissipating group	18	0.9134 ± 0.3137

Statistical analysis result: t value =-3.627, P value =0.001, two groups has significant difference (P<0.05).

**Table 9.** Nasopharynx cancer tissue HA mRNA relative gray value pathological indicators and clinical parameter relationship.

Group	N	HA content ( $\bar{X} \pm S$ )	Statistical analysis result	
			t-value	P-value
<b>Age</b>				
≤ 61	41	0.9409 ± 0.4919	1.194	0.238
>61	9	0.7404 ± 0.4488		
<b>Gender</b>				
Male	26	0.7914 ± 0.4998	0.237	0.812
Female	24	0.7603 ± 0.4186		
<b>T grade</b>				
T1-2	25	0.7787 ± 0.4753	0.034	0.973
T3-4	25	0.7742 ± 0.4504		
<b>Tumor differentiation</b>				
High and medium Differentiation	10	0.5247 ± 0.3938	2.072	0.044
Low differentiation	40	0.8464 ± 0.4487		

In addition to tumor differentiation of HA mRNA in high and medium differentiation and low differentiated between the two groups is significant (P<0.05), the remaining parameters are not significant.

**Nasopharynx cancer HA mRNA expression**

Nasopharynx cancer HA mRNA (RT-PCR) expression N(+) group, cancerous tissue expression is higher than that of N(0) group (P <0.05), three months after radiotherapy, not complete dissipating group is higher than that of complete dissipating

group (P<0.05). Relative contents of CD44 expression in cancer tissues compared with internal reference bands are shown in Tables 7-9.

**Discussion**

Metastasis is the main cause of treatment failure and death of patients with malignant tumor. Revealing the mechanism of tumor metastasis and predicting tumor metastatic potential are helpful for determining prognosis and selection of reasonable and effective treatment plans and for exploring more specific and effective anti-tumor drug clinical application methods so as to prolong survival and improve quality of life, which is a top priority of the current cancer research. Nasopharynx cancer can easily cause cervical lymph node metastasis, 40-50% patients seek treatment with neck metastasis and more than 70% patients suffer cervical lymph node metastasis, lymph node metastasis is an important factor affecting the efficacy and prognosis of Nasopharynx cancer. Therefore, doing intensive study of cervical lymph node metastasis mechanism has great significance for early diagnosis and prevention of Nasopharynx cancer lymph node metastasis and improving survival and quality of life.

As a glycoprotein crossing cell membrane, CD44 gives strong growing and metastasis ability through adhesion with hyaluronic acid, collagen and vascular endothelial cells etc. in extracellular matrix, and plays an important role in growth and development of tumor. The relationship between CD44 and tumor metastasis was confirmed by German researchers in 1991[4]. At present, most studies [5] consider that CD44 has close relationship with occurrence, development, invasion and metastasis of malignant tumor. By detecting a variety of CD44 molecules in malignant tumors, it is found that many tumors have different degrees of CD44 molecule expression, and has close relationship with tumors growth, development, metastasis and prognosis. Lu et al. [6] found that in cervical adenocarcinoma, either preinvasive carcinoma or invasive cancer has CD44s expression, and the latter is significantly higher than that of the former. According to many reports, the higher is CD44 expression, the stronger is the lymph node metastasis [7], Ni et al hold that CD44 can be used as a gastric cancer metastasis associated marker in clinical testing, providing prognostic and therapeutic reference for clinicians. Wang et al, also reported that CD44 protein expression is related to laryngeal carcinoma and can be an important indicator for predicating laryngeal cancer metastasis and prognosis. Weber et al [8] studied the function of CD44 in tumor metastasis from gene level, and finds that though tumors in mice whose CD44 gene has been removed can survive but cannot metastasize, which once again proves the important role of CD44 in the regulation of tumor metastasis. Recently, there are also negatively related or unrelated reports, Wang et al reported that CD44v6 protein expresses in downward

adjustment way during nasopharynx cancer metastasis, its normal expression may prevent tumor infiltration of surrounding tissue and lymph nodes. Liutz et al. think that CD44 expression has no relation with ovarian cancer progression and metastasis [9].

The growth and metastasis of tumor is a complex process with many steps which influenced by many factors. People have studied on the mechanism of tumor's genesis and development in depth and in detail. Few years ago, they have already noticed the formation mechanism of the mesenchyma [10]. The study have shown that HA compounds from the plasma membrane of the mesenchymal cells. Then it released as a soluble product, passed into the blood from the lymph circulation and took part in the formation of PG, which is the basis of the ground substance of the connective tissue. The ground substance around the tumor with strong invasiveness contains rich HA. In contrast, the tumor with poor invasiveness hardly contains any HA [11]. The HA synthesis in the ground substance around the tumor help the tumor to extend into the surrounding tissue [12]. The fibroblast in the tumor cells and the mesenchyma around the tumor can produce and accumulate HA. Many kinds of solid tumor such as prostate cancer, thyroid cancer, breast cancer, ovarian cancer and glioma have excessvie expression of HA phenomenon. HA related to the adhesion of nasopharyngeal carcinoma cell and the change of cytoskeleton [13]. Nasopharyngeal carcinoma cells enhanced their ability of proliferation, invasion and metastasis through adhering with HA.

There is not so much such research in the research of nasopharyngeal carcinoma. There is only the negative report on Wang Wei's CD44 variant of CD44v6 protein. Our research respectively studied, from the level of protein and gene level, the relationship between the expression of CD44 in nasopharyngeal carcinoma tissue and the clinical and pathological indicators (including age, sex, tumor size, pathological grading, lymph node status, whether lymph nodes metastasis fade away or not after radiotherapy). The result is that the expression of CD44 has statistically significant difference between lymph node metastasis or not group and lymph node fading or not after radiotherapy group: CD44 protein positive expression rate and relative content of mRNA in lymph node metastasis group is significantly higher than that in lymph node not metastasis group. After radiotherapy, CD44 protein positive expression rate and relative content of mRNA in lymph node not completely subsided group is significantly higher than that in lymph nodes metastasis group. As for the relative content of CD44 mRNA, synthesis of a group of high and senior differentiation have  $P < 0.05$ , significant difference when compared with low differentiation. As for the positive expression rate of CD44 protein, synthesis of a group of high and senior differentiation have  $P > 0.05$ , which is not a significant difference when compared with low differentiation but close to 0.05, could not be high, medium and low by comparison. Because there is very little nasopharyngeal carcinoma high differentiation, it could not be compared alone on high, medium and low. The results indicated that in the clinical pathologic indexes in patients with nasopharyngeal

carcinoma (NPC), CD44 either at the genetic level or at the protein level is closely related to whether lymph node metastasis and the sensitivity of radiotherapy of metastasis lymph nodes. On gene level it associated with the pathological differentiation while on protein level it related with the trend of pathological differentiation. High expression of CD44 suggests the easy happening nasopharyngeal tumor lymphatic metastasis and metastasis of lymph nodes have poor sensitivity to radiotherapy.

The cancer treatment of CD44 antibody is still in the stage of laboratory. It has already reported [14] that CD44 McAb can restrain the proliferation of multiple myeloid leukemia cell lines and induce their differentiation. In vitro experiments it has been found that CD44 McAb and bone bridge McAb can obviously decrease the susceptibility to cancer killer cells destruction from tumor cells. Our research results suggest that CD44 play an important role in the process of invasion and metastasis of nasopharyngeal carcinoma (NPC). It can be used as related markers of nasopharyngeal carcinoma metastasis in clinical testing to provide valuable reference for clinical doctors to judge prognosis and formulate a more effective treatment. It is expected to play a huge role in nose pharynx cancer prevention, control and evaluation of prognosis.

The specific combination of HA and CD44 molecules adhered on the surface of tumor cell changes the adhesion force and enhances aggressive and transfer power. Organ lymph node is the organ where the tumor spread first arrived, which contains a lot of HA, and is the main part of HA metabolism. In recent years, the research found that the interaction of CD44 - HA is associated with the spread of the tumor cells. CD44 has absorption and degradation effect on HA. This function can not only make tumor cells avoid adhesion with other molecules under the condition of higher HA content, but also can make it attack tumor cells with higher HA content [15], contribute to tumor cells across through the basement membrane and cell wall, and eventually complete transferring itself. The expression of CD44 is positively related to the tumor metastasis. Our results also confirmed this.

The latest research reported that tumor cells can generate a kind of protein which can deliver drugs out of the cell. HA combined with its CD44 receptor along with phosphoinositide 3-kinase urge tumor cells to increase the protein synthesis, thus make tumor cells become resistance to drugs [16]. Therefor it can make use of antagonism material to interference the combination of HA and CD44 receptor protein, thus it enhanced the sensitivity of tumor cells to chemotherapy drugs, which is conducive to the treatment of tumor. Through the experiment, Thomas [17] proves that the adhesion and metastatic is greatly increased after HA combined with CD44 in melanoma cells in the human body. To utilize CD44 antibodies or CD44 competition adhesion molecules can inhibit CD44 from combining with HA, thus inhibiting the improvement of the adhesion and metastatic, Zeng [18] use oligosaccharides to fight against HA and the reactivity of its receptor CD44 and inhibit the growth of the rats in vivo melanoma. Knudson [19] research and indicates that, in intact

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cells, the chondroitin sulfate can competitively combine with HA receptor. So we can antagonize the combination of HA and CD44 through accessing HA competitive polysaccharide into human. At present, interference to the combination of HA and CD44 mainly include: (1) sealing HA receptor; (2) prevent the combination of HA and CD44, decompose the combination and correct combination way; (3) gene therapy; (4) blocking CD44, change the CD44 configurations.

This essay discussed the research on the lymphatic metastases of HA and CD44 in nasopharyngeal carcinoma and their radiation sensitivity respectively in protein level and molecular biology level. The results showed that the expression of HA and CD44mRNA in nasopharyngeal carcinoma have significant difference between whether in lymph node metastasis group and lymph node fading after radiotherapy group. The relative content of protein of HA, CD44 mRNA and CD44 in group with lymph node metastasis is significantly higher than the group without lymph node metastasis. The relative content of protein of HA, CD44 mRNA and CD44 in lymph node not completely subsided after radiotherapy group is significantly higher than the lymph nodes after radiotherapy group.

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### **Corresponding to:**

De Yu Sun

Department of Radiology

Dalian Medical University

PR. China