

## Original Article

# Over-expression of p53, p21 and Cdc2 in histologically negative surgical margins is correlated with local recurrence of laryngeal squamous cell carcinoma

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**Abstract:** The aim of this study was to explore the relationship between the expression of p53, p21 and Cdc2 in the early laryngeal cancer with negative pathological margins and its local recurrence. During 2004-2010, a total of 85 patients with early laryngeal cancer were selected in Tangshan Union Hospital, Hebei, China, and immunohistochemical method was used to detect the expression of p53, p21 and Cdc2 in the negative pathological margin tissues. All patients were followed up for two years to collect pathological data for evaluating the survival and tumor recurrence. Two years after surgery 14 of 85 patients with laryngeal cancer presented with recurrence (recurrent group), while 71 patients without recurrence (none recurrent group). The positive rate of p53, p21 and Cdc2 protein in laryngeal cancer tissues was 60.0% (51/85), 38.8% (33/85) and 70.6% (60/85), respectively, while that of the three proteins in the cancer adjacent tissues was 36.5% (31/85), 21.2% (18/85) and 29.4% (25/85), respectively. The differentiation and TNM stage of tumor had no correlation with the three proteins. The positive rate of p53 in the surgical margin of the recurrent group and non recurrent group was 71.4% (10/14) and 29.6% (21/71) ( $P = 0.003$ ), that of p21 was 50.0% (7/14) and 15.5% (11/71), ( $P = 0.011$ ) and Cdc2 was 57.1% (8/14) and 23.9% (17/71) ( $P = 0.030$ ), respectively. In conclusion, p53, p21 and Cdc2 may be involved in the occurrence, development and recurrence of laryngeal squamous cell carcinoma. Overexpression of p53, p21 and Cdc2 in the surgical margin of early laryngeal cancer is closely related to local recurrence of tumor.

**Keywords:** Laryngeal squamous cell carcinoma, p53, p21, Cdc2, immunohistochemistry, local recurrence

## Introduction

Laryngeal squamous cell carcinoma is the most common histological type of head and neck cancer, its occurrence and development is a multi-factor, multi-stage process. Of all the factors that affecting the progressing of laryngeal cancer, the cell cycle proteins may play a more important role in the development of the tumor. The cell cycle is the cascade events that promote a growing cell to duplicate all its components to split into two daughter cells. In all the regulators that control the progression of cell mitosis, the cyclins and cyclin-dependent kinases (CDKs) are the two critical classes of molecules. These proteins form a heterodimer in which cyclins are the regulatory subunits and CDKs are the catalytic subunits; when the com-

plex is activated from external signals, CDKs activate or inactivate downstream target proteins to orchestrate coordinated entry into the next phase of the cell cycle. CDK is divided into two categories which are activated by cyclin CDK binding protein or by CDK inhibitors CKIs. CKI inhibitors are divided into two families: one is INK4 family, including p15<sup>INK4B</sup>, p16<sup>INK4A</sup>, p18<sup>INK4C</sup> and p19<sup>INK4D</sup>, which can bind CDK4 and CDK6 specifically; the other is the kinase inhibitor protein (KIP) or CDK interacting proteins family (CIP), including p21, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>, all these CKIs can binds to and inhibits cyclin proteins binding [1, 2]. Cyclin-dependent kinase 1 (CDK1) is a member of the Ser/Thr protein kinase family coded by cell division cycle (cdc) gene 2 whose relative molecular weight is 34KD. In the late stage of G2, Cdc2 and cyclin B

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**Table 1.** Expression of p53, p21 and Cdc2 proteins in laryngeal carcinoma tissue (No. of patients)

Clinical Features	Total No.	p53		P	p21		P	Cdc2		P
		+	-		+	-		+	-	
Total No.	85	51	34		33	52		60	25	
Differentiation										
Well-moderately Differentiated	63	35	28	0.157	23	40	0.459	42	21	0.179
Poorly differentiated	22	16	6		10	12		18	4	
TNM staging										
Stage T1a	58	32	26	0.183	20	38	0.229	40	18	0.630
Stage T1b	27	19	8		13	14		20	7	

combined into Cdc2-cyclinB kinase complex, which is called mitosis promoting factor or maturation promoting factor (MPF), can prompt cells from G2 phase into M phase [3]. Less report was found about the alterations of p53, p21 and Cdc2 in laryngeal carcinoma currently. We thus used immunohistochemical method to detect the expression of p53, p21 and Cdc2 proteins in 85 cases of laryngeal cancer and adjacent tissues to analyze its relationship of the pathological changes and prognosis of tumor.

### Material and methods

#### Cases description

The study involved 85 patients who underwent CO<sub>2</sub> laser surgery at Tangshan Union Hospital, China, from 2004 to 2010, Tissues 5 mm away from cancer were used as control. The patients aged from 48 to 79 years with a mean age of 52.3 ± 9.8 years, including 78 males and 7 females, and according to the AJCC Cancer Staging, they were all belonged to stage I that was subdivided into T1a and T1b, including 58 cases in stage T1a and 27 cases in stage T1b. All patients were treated without radiotherapy and chemotherapy before surgery. Pathological classification of tumor was graded as well-, moderately- and poorly-differentiated squamous cell carcinoma of the larynx. The study was approved by the Ethics Committee of Tangshan Union Hospital.

#### Immunohistochemical staining

We studied the correlation of the proteins to tumor recurrence by detecting the expression of p53, p21 and Cdc2. All specimens obtained from patients were fixed in 10% neutral formalin, embedded in paraffin routinely and one rep-

resentative block from each patient was sectioned at 4 μm, stained with hematoxylin and eosin (HE) and evaluated by immunohistochemistry according to the protocol described in the manufacturer's guide accompanying the kit. Known positive expression samples of p53, p21 and Cdc2 were used as positive immunostaining controls. For the negative control, the primary antibodies were replaced with phosphate-buffered saline (PBS). The mouse monoclonal antibody against human p53 (Santa Cruz, USA, 1:100) and Cdc2 were purchased from Santa Cruz Biotechnology (Santa Cruz, USA, 1:300), and p21 was obtained from Invitrogen Biotechnology (Invitrogen, USA, 1:100). SP immunohistochemistry kit was purchased from New Biotechnology Development Co, Fuzhou, China.

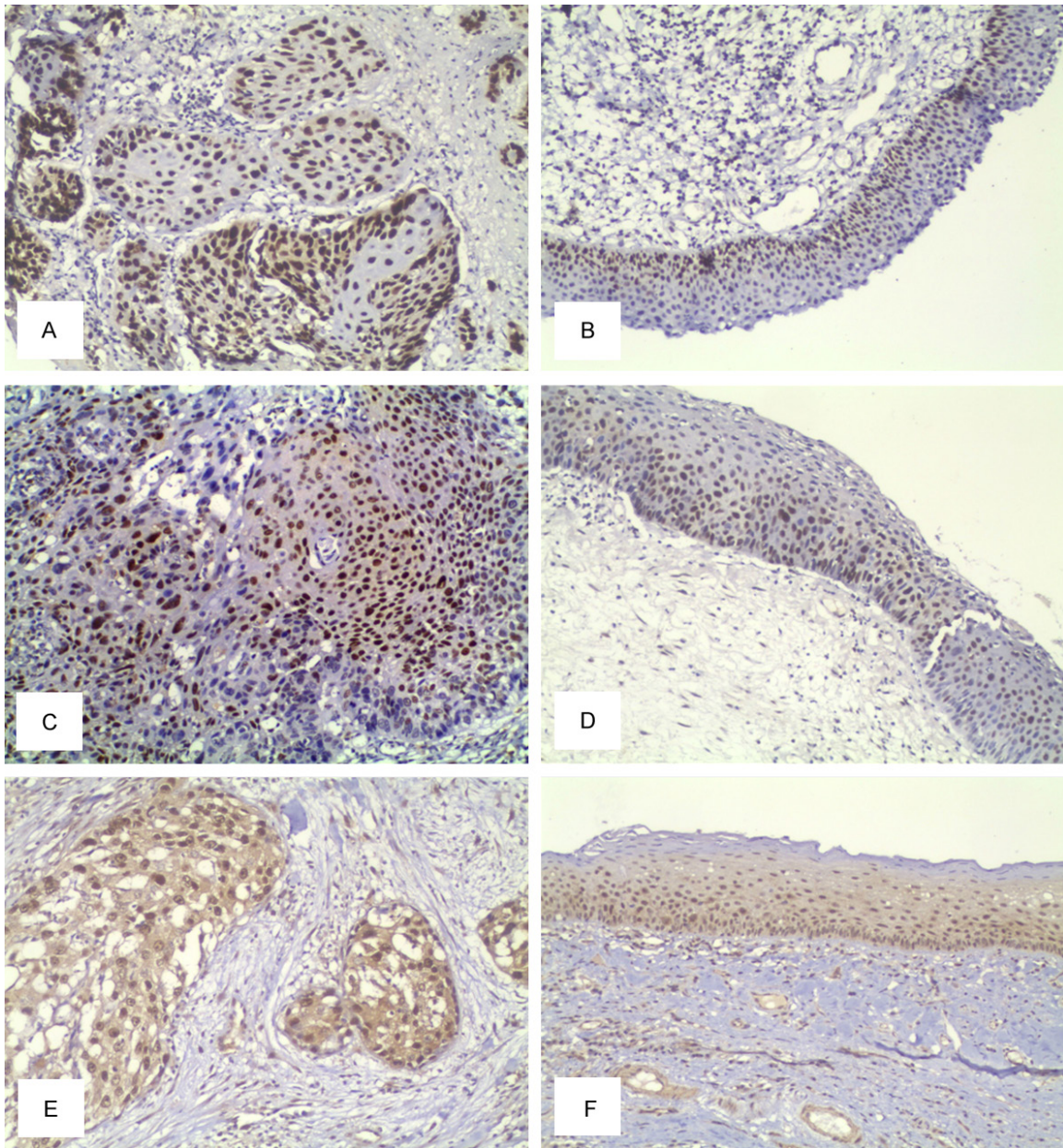
#### Evaluation of immunohistochemical analysis

Positive signals of the immunohistochemical staining were brown or brownish yellow in color. Nuclear staining was considered positive on the immunostaining for p53, and the cytoplasmic and/or nuclear immunostaining were considered positive for Cdc2 and p21. Staining was observed in 10 representative high-power fields, the brown nuclear and/or cytoplasmic staining cells were observed and counted [4-7]. Positive staining in more than 10% of the cells was considered positive, photographed under × 100 magnification, while less than 10% or colorless were defined as negative. The scoring results were divided into two categories, either negative or positive. The uninterpretable results were eliminated from further consideration.

#### Statistical analysis

The statistical analyses were performed with PASW Statistics 20.0 (SPSS Inc., Chicago, IL,

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**Figure 1.** Expression of p53, p21 and Cdc2 in laryngeal squamous cell carcinoma and its surgical margins. A. Immunohistochemical detection of p53 in laryngeal carcinoma. B. Immunohistochemical detection of p53 in surgical margin of laryngeal tumor tissue. C. Immunohistochemical detection of p21 in laryngeal carcinoma tissue. D. Immunohistochemical detection of p53 in surgical margin of laryngeal tumor tissue. E. Immunohistochemical detection of Cdc2 in laryngeal carcinoma tissue. F. Expression of Cdc2 in surgical margin of laryngeal tumor tissue. SP method, original magnification  $\times 100$ .

USA). The relationships between the expression of p53, p21 and Cdc2 and the various clinicopathological findings were evaluated using the Chi-square test, and the Spearman Chi-square test rank correlation between the expressions of two proteins. *P* values less than 0.05 were considered to be statistically significant.

### Results

#### *Clinical and follow-up data of patients with early laryngeal cancer*

The clinical data of the patients and the relationships between the clinicopathologic parameters and the p53, p21 and Cdc2 expression in

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**Table 2.** Relationship between the expression of p53, p21 and Cdc2 and prognosis in laryngeal tumor resection marginal tissue (No. of patients)

Prognosis	Total No.	p53		P	p21		P	Cdc2		P
		+	-		+	-		+	-	
Recurrence	14	10	4	0.003	7	7	0.011	8	6	0.030
Non recurrence	71	21	50		11	60		17	54	

**Table 3.** Relationship between expression of p53 and p21 in laryngeal tumor resection marginal tissue (No. of patients)

	p53 (+)	p53 (-)	P
p21 (+)	8	10	0.429
p21 (-)	23	44	

**Table 4.** Relationship between expression of p21 and Cdc2 in laryngeal tumor resection marginal tissue (No. of patients)

	p21 (+)	p21 (-)	P
Cdc2 (+)	7	18	0.320
Cdc2 (-)	11	49	

85 early laryngeal cancer are summarized in **Table 1**. Three pathologists examined the cases independently and if there was any disagreement, we had a discussion with another pathologist to reach consensus. Pathologists were responsible for the postoperative follow-up. Among 85 laryngeal cancer patients followed up for 2 years there were 14 cases of local recurrence (recurrence group) while 71 patients without recurrence (recurrence-free group). As patients in the group of laryngeal cancer were all stage T1 without lymph node metastasis, there was no correlation of the expression of the three proteins with tumor differentiation or TNM stage (**Table 1**).

### *Correlation of the expression of p53, p21 and Cdc2 with outcomes of patients with laryngeal carcinoma*

The expression rates of p53 protein in laryngeal carcinoma and its surgical margin were 60.0% (51/85) and 36.5% (31/85) respectively (**Figure 1A, 1B**), whereas those of p21 protein were 38.8% (33/85) and 21.2% (18/85) (**Figure 1C, 1D**) and Cdc2 were 70.6% (60/85) and 29.4% (25/85), respectively, (**Figure 1E, 1F**). The p53 expression rate was 71.4% (10/14)

and 27.9% in the cases of recurrent and none recurrent groups in the surgical margins of the cancer, respectively, with statistically significant difference ( $P = 0.003$ ), whereas those of p21 were 50.0% (7/14) and 15.5%

(11/71), respectively, and these difference was also statistically significant ( $P = 0.003$ ). Furthermore, the positive rates of Cdc2 expression were 57.1% (8/14) and 23.9% (17/71) in the cases of recurrent and none recurrent group in the surgical margins of the cancer, respectively, with statistically significant difference ( $P > 0.05$ ) (**Table 2**). There was no correlation between the expression of p53 and p21, and between p21 and Cdc2 in the surgical margins of the cancer ( $P > 0.05$ ) (**Tables 3, 4**).

### **Discussion**

CO<sub>2</sub> laser is one of the most common treatment modalities for early laryngeal cancer, especially for the patients of stages T1 and T2. The goals of treatment are preserving laryngeal voice and avoiding the pain of surgical laryngeal splitting so as to improve patients' quality of life [4]. Additional goals include minimizing the risk of serious complications and cost of treatment. Recently the 5-year survival rate of laryngeal cancer was ranged from 40% to 80%. Recurrence after surgery is one of the main factors that affecting prognosis and whether the surgical margin had residual tumor tissue or not was significantly associated with recurrence. Local recurrence rate in patients with laryngeal negative margin of surgical pathology is rather high [5]. So molecular biology research about the laryngeal carcinoma and its surgical marginal tissue is still a hotspot nowadays.

Wild-type p53 tumor suppressor gene is one of the most common tumor suppressors that plays roles of suppressing tumor growth by a variety of ways and known for its "molecular policeman" by maintaining the stability of the human genome. In contrast, mutant p53 gene plays a role of proto-oncogenes who can promote the occurrence and development of tumors. Mutation of p53 gene was found in almost all human tumors. Wild-type p53 protein can form a tetramer *in vivo* and its half-life

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is only 6-20 minutes, thus it cannot be detected by immunohistochemistry. While half-life of mutant p53 protein was prolonged; and then it can be detected by immunohistochemistry [6]. It has been reported that positive rate of p53 protein in primary laryngeal squamous cell carcinoma was 40-60%, which was the most closely related oncogenes discovered by so far with the occurrence of laryngeal carcinoma and correlated significantly with the degree of malignancy of laryngeal cancer [7]. p53 protein was strongly positive in laryngeal squamous cell carcinoma tissues and was also detected in adjacent tissues of the tumor. Other study suggested that pathologists should detect p53 protein in the surgical margins routinely after surgery that was meaningful for the determination of local tumor recurrence [8]. Jalali MM *et al* [9] have shown that p53 expression is associated closely with laryngeal cancer recurrence; the recurrence and mortality rate of p53 positive group was significantly higher than that of the negative group. In this study, the positive rate of p53 protein in laryngeal cancer and surgical margin of tumor adjacent tissues was 60.0% (51/85) and 36.5% (31/85), respectively. In recurrent group and non recurrent group of this cohort, the positive rate of p53 protein in the cancer surgical margin was 71.4% (10/14) and 29.6% (21/71) ( $P = 0.003$ ), respectively. Therefore, it is implicated that detecting the expression of p53 protein in the laryngeal surgical margin can predict the risk of tumor recurrence.

p21 is one of the CIP family members of CKI, and it can inhibit the cell cycle progression mainly through inhibition of the function of CDK2. Relationship of p21 gene and tumor is mainly synergies with other related genes in the expression levels, which acts in a p53-dependent pathway or p53-independent pathway [10]. Clinical reports of p21 expression and tumor prognosis are not identical. Kapranos *et al* [11] have shown that p21 expression is a better prognostic factor in stage III head and neck squamous cell carcinoma. Bandoh *et al* [12] studied the expression of p21 in 70 cases of squamous cell carcinoma of the maxillary sinus and found that the missing of p53-dependent p21 expression was found in patients with high proliferative tumor cells. Kaplan-Meier analysis showed that the expression of p21 was not correlated with longer dis-

ease-free survival of patients. Fischer *et al* [13] studied the relationship between the expression of p21 and prognosis in 144 cases of hypopharynx squamous cell carcinoma and found that p21 expression was correlated with clinical lymph node staging (positive rate of p21 in group N0 and group N1-2 was 66.7% and 93.7%, respectively;  $P < 0.001$ ), local recurrence (p21 positive rate of recurrence group and non-recurrence group was 69.6% and 50%, respectively;  $P = 0.031$ ) and poor prognosis (5-year survival rates of p21 positive group and p21 negative group were 35.3% and 54.7%, respectively;  $P = 0.016$ ). Expression of p21 is an important marker of poor prognosis in head and neck squamous cell carcinoma (HNSCC). Our study found that the recurrence rate in patients with p21 positive expression is higher than that of patients with negative one, and the difference was statistically significant. Analysis shows that expression of p21 has two pathways: p53-dependent and p53-independent. It is a complicated relationship between the expression of p21 and p53 in tumor tissues, and the expression of p21 and p53 was heterogenous in different cells and tumors [14]. p21 expression was heterogeneous in various tumors, so that it is still controversial that p21 is a tumor suppressor gene or an oncogene. Early studies have shown that when p21 was answering a variety of environmental stimuli, cell cycle arrests, which supported the opinion that p21 is a tumor suppressor gene. In addition, biochemical and genetic studies have shown that p21 does not rely on classical signaling pathway of p53, and acts as a major effector of tumor suppressor genes in a variety of antiproliferative activities. Despite its main role is to inhibit cell proliferation and promote cell differentiation and senescence, but new studies have shown that under certain circumstance p21 can promote cell proliferation and plays a carcinogenic role. Thus, p21 is often not expressed in various human tumors and its expression depends on the cell environment, in which p21 may act as a tumor suppressor gene, or an oncogene [10, 15]. In laryngeal squamous cell carcinoma, p21 protein expression may be in p53-independent pathway, which increased the stability of p21 protein and accumulated protein rather than increasing transcription.

Cde2 is one of the most important regulated kinase of cell cycle and its activity is not only

regulated positively by cyclin B1, but also by CKI negatively [16]. The main function of Cdc2 is to monitor spindle microtubule assembly and kinetochore's proper connection of DNA in the G2/M checkpoint. If an error occurs in adjustment mechanism of Cdc2, it will lead directly to cell differentiation disorders, disorder of cell cycle progression and induce abnormal cell proliferation or malignant transformation, thus promoting the occurrence and progression of tumor [17-19]. Cdc2 overexpressed in many cancers, such as oral squamous cell carcinoma [20], tongue squamous cell carcinoma [21], supraglottic cancer [22], esophageal squamous cell carcinoma, esophageal adenocarcinoma [23], gastric cancer [24], liver cancer [25], colorectal cancer [26], breast cancer [27], ovarian cancer [28] and so on. This study found that positive rate of Cdc2 protein in laryngeal cancer and cancer adjacent surgical margin tissues was 70.6% (60/85) and 29.4% (25/85), and in laryngeal recurrent group and non-recurrent group, the positive rate of Cdc2 in the cutting edge of cancer is 57.1% (8/14) and 23.9% (17/71), respectively. Therefore we believe that expression of Cdc2 protein is closely related to recurrence and poor prognosis of laryngeal cancer.

HNSCC patients with positive pathological margins has high postoperative recurrence rate, about 75% of local recurrence. While for patients with negative pathological margins there are still 25% of patients with local recurrence ultimately [29]. Therefore, some researchers conducted molecular studies for the negative pathological margin to clarify the underlying mechanism. It is considered that two mechanisms could explicate the local recurrence with the negative pathological margin after surgery: one is the possible presence of residual tumor cells, which means the incomplete cutting edge. That histological technology nowadays could not find the residual disease is called minimal residual cancer (MRC). Another is the presence of the lesion that may have changed at the molecular level (precancerous lesions), and eventually turn into invasive cancer. Therefore we used various techniques including immunohistochemistry, RT-PCR, FISH and other molecular studies to identify potential molecular markers of local recurrence. Studies found that local recurrence of tumor was related to the expression of p53, CCDN1,

p16, etc [30]. But little about the combined detection of protein expression in laryngeal peripheral tissues have reported yet.

In conclusion, the expression of p53, p21 and Cdc2 protein in laryngeal squamous cell carcinoma was higher than that in tumor adjacent tissues, while the local recurrence of laryngeal squamous cell carcinoma was significantly correlated with positive expression of proteins in tumor adjacent tissue, suggesting that expression change of p53, p21 and Cdc2 protein is an important factor affecting the occurrence of laryngeal carcinoma. p53, p21, and Cdc2 are expected to become important biomarkers for evaluation of prognosis and treatment of laryngeal squamous cell cancer. For patients with positive expression of p53, p21 and Cdc2 should be closely followed up, shorten the interval of follow-up time and once potential recurrence suggested, prompt surgical should be made to remove of the tumor again. Immunohistochemistry is a simple, sensitive and reliable method to detect the expression of p53, p21 and Cdc2 jointly in early laryngeal cancer and its surgical margins. Combined detection of the three proteins may be a useful reference for screening patients at high risk of recurrence which is worthy of further molecular studies.

### Disclosure of conflict of interest

None.

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