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RESEARCH PAPER

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Expression and Significance of N-myc downstream regulated gene 2 in the process of Esophageal Squamous Cell Carcinogenesis

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ABSTRACT

It has been reported that the expression of tumor suppressor gene N-myc downstream-regulated gene 2 (NDRG2) was significantly reduced in human solid tumors, including esophageal squamous cell carcinoma (ESCC). This study aimed to explore whether the difference of NDRG2 expression exists in different stages of ESCC and provides a basis for the early diagnosis and prognosis of ESCC. Immunohistochemical staining was used to investigate the expression level of NDRG2 in samples from 91 patients with mild-to-moderate dysplasia, early ESCC, and advanced ESCC. The relationship between the expression of NDRG2 and clinicopathological characteristics of the patients was analyzed. The results showed that positive expression rates of NDRG2 in tissues adjacent to early ESCC (76.7%), or from mild-to-moderate dysplasia (74.1%), and early ESCC (83.3%) were significantly higher than in tissue from advanced ESCC (55.9%). The positive expression rate in advanced ESCC was significantly lower than in the other three tissue types ($p < 0.05$). There was a significant difference ($p < 0.05$) and correlation (Cramer's $V = 0.351$, $p = 0.019$, <0.05) between the expression of NDRG2 and the clinical stage in the 64 patients with ESCC. In conclusion, this study found that the expression of NDRG2 gradually decreased with the progression of esophageal lesions into advanced ESCC. This difference in positive expression rate was more obvious in male patients and patients under 60 years of age. Therefore, the detection of NDRG2 plays an important role in differentiating early ESCC from advanced ESCC.

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Introduction

Esophageal cancer is one of the most common malignancies in the world and, according to GLOBOCAN 2012, has the sixth highest cancer-associated mortality rate worldwide [1]. Histologically, esophageal carcinoma consists mainly of squamous cell carcinoma, which accounts for more than 90% of all esophageal cancers, and adenocarcinoma. Squamous cell carcinoma is the dominant form of esophageal cancer in Asian countries, including China. Due to the lack of typical early clinical symptoms, most patients with esophageal squamous cell carcinogenesis (ESCC) are not diagnosed until the disease is at an advanced stage, and their 5-year survival rate is less than 20% [2]. With early diagnosis and treatment, the 5-year survival rate may reach more than 90%.

Early ESCC diagnosis is important but remains difficult. Patients with early ESCC often lack specific symptoms, which can easily be missed. Endoscopy combined with pathological examination is the best way to diagnose early ESCC, but many patients reject this examination because it is uncomfortable. Thus, endoscopic screening is still not widely carried out in most parts of China. Currently, specific tumor markers to diagnose early ESCC do not exist, so identifying simple and effective ESCC markers that enable early ESCC diagnosis is an important research goal.

While the pathogenesis of ESCC has not been fully elucidated, a large number of molecules appear to be involved in its development. The N-myc downstream-regulated gene 2 (NDRG2), which was discovered by Chinese scholars in the Fourth Military Medical University, is thought to be a potential tumor suppressor gene. Some studies [3,4] show that NDRG2 expression is down-regulated in ESCC, and suggest that it might

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suppress tumor growth. However, these studies are based only on patients with advanced ESCC and do not include patients with mild-to-moderate dysplasia of esophageal squamous cells or early ESCC. The current study included three groups of patients, who had mild-to-moderate dysplasia of esophageal squamous cells, early or advanced ESCC.

This study aimed to explore whether the difference of NDRG2 expression exists in different stages of ESCC and provides a basis for the early diagnosis and prognosis of ESCC.

Materials and methods

Patients and collection of samples

Thirty-four cases of advanced ESCC, 30 cases of early ESCC, and 27 cases of mild-to-moderate dysplasia of esophageal squamous cells were randomly selected from the patients who visited the Department of Gastroenterology and Thoracic Surgery of Beijing Friendship Hospital between January 2013 and September 2017. Patients' characteristics, such as gender, age, location of tumor, stage of disease, and histopathological factors were obtained from the medical records. All the patients with ESCC were diagnosed with clinical and pathological features. Specimens from most patients with early ESCC and from some with mild-to-moderate dysplasia came from tissues resected by endoscopic submucosal dissection, and diagnostic accuracy was reliable. For all patients, pathological tissue sections were obtained from the Pathology Department of Beijing Friendship Hospital for immunohistochemical staining. TNM staging and G classification were performed according to the American Joint Committee on Cancer/Union for International Cancer Control Classification Guidelines (8th edition) [5], and carcinoma in situ was classified as early esophageal cancer on the basis of the Japanese Classification of Esophageal Cancer (11th edition) of the Japan Esophageal Society [6]. Early ESCC was defined as carcinoma cells limited to the mucosal or submucosal tissues, while advanced ESCC was defined as infiltration of carcinoma cells beyond the submucosal tissues. The study was approved by the Ethics Committee of Beijing Friendship Hospital.

Immunohistochemical staining

Immunohistochemical staining was performed to assess NDRG2 protein expression. Formalin-fixed tumor tissues were embedded in paraffin, and serial 4 μm sections were obtained using a Leica microtome. For staining, tumor sections were deparaffinized in toluene, rehydrated in an alcohol gradient and permeabilized in Tris-ethylene-diaminetetraacetic acid (EDTA) buffer (pH 9.0). Sections were then incubated with endogenous peroxidase blockers (PV-6000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) for 10 min at room temperature to block endogenous peroxidase activity, after which they were washed in phosphate-buffered saline (PBS). Nonspecific binding was blocked by incubation with normal goat serum for 30 min at room temperature. Sections were incubated with mouse anti-NDRG2 monoclonal antibody (1:100; Abcam, UK) at 4°C overnight, followed by NDRG2 antibody incubation with a horseradish peroxidase-labeled goat anti-mouse/rabbit IgG polymer (PV-6000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, China) for 1 h at room temperature. After washing, sections were incubated with 3,3 -diaminobenzidine (DAB) color developing solution, lightly counterstained with hematoxylin, and observed under a photomicroscope.

Immunohistochemical analysis

Sections without the primary antibody were used as negative controls. The slides were evaluated independently by two pathologists who were blind to the study. When there was disagreement, a decision was reached after joint discussion. The score of NDRG2 staining intensity was determined by comparison with the background color and defined as follows: 0 = colorless, 1 = light yellow, 2 = deep yellow, 3 = tan. The score for the percentage of positive cells was determined by applying the following rules: 0 = <2%, 1 = 2%–25%, 2 = 26%–50%, 3 = 51%–75%, and 4 >75%. Immunohistochemical staining results were determined by combining the staining intensity with the number of positive cells. Finally, we determined the overall results by calculating the product of the above two scores: 0–1 = -, 2–4 = +; 5–

8 = ++, and ≥9 = +++. In the present study, – or + were regarded as negative and ++ or +++ as positive.

Statistical analysis

Statistical analysis software SPSS17.0 was used to analyze the data in the study. Continuous data were expressed as a ratio or percentage, and comparisons between groups were performed by the Chi-square (χ^2) test. Fisher's exact test was used for the analysis of data when the minimum theoretical frequency was less than 1, or the total sample size was less than 40. The continuity correction χ^2 test was used for the analysis of data when the total sample size was greater than 40, and the minimum theoretical frequency was between 1 and 5. For an χ^2 test of $R\times C$ table data, the following conditions needed to be satisfied: the minimum theoretical frequency was ≥1, and the ratio of theoretical frequency between 1 and 5 could not be more than one fifth of the total number. Correlation analysis of count data was carried out with the χ^2 test, Phi coefficient φ and Cramer's V test. A p value of <0.05 was accepted as statistically significant. Compa risons of data between groups were made by t-test for comparison of means of two independent samples.

Results

The purpose of this study was to investigate the difference of NDRG2 gene expression in different stages of ESCC. In this study, we first confirmed the differences in NDRG2 gene expression in each disease stage of ESCC, and then conducted subgroup analysis and found that such differences were more obvious in some populations.

Patients' clinicopathological characteristics

The selected patients included 18 females and 73 males, who had a mean age of 63.3 years (range, 43–82 years). The patients' characteristics are summarized in Table 1.

Relationship between clinicopathologic characteristics and expression of NDRG2 in the patients

As shown in Table 2, there was no significant difference between the expression of NDRG2 and gender, age, drinking, and smoking habits, or the location of esophageal lesions in the 91 patients included in this study ($p > 0.05$) and no significant difference between the expression of NDRG2 and histological tumor type in the 64 patients with ESCC ($p > 0.05$). However, there was a significant difference ($p < 0.05$) and correlation (Cramer's V = 0.351, p = 0.019, <0.05) between the expression

Table 1. Patients'clinicopathological characteristics.

Variables		Mild-to-moderate dysplasia(%)	Early ESCC(%)	Advanced ESCC(%)
Gender	Male	18(24.6)	28(38.4)	27(37.0)
	Female	9(50.0)	2(11.1)	7(38.9)
Age (years)	≥60	17(28.3)	19(31.7)	24(40.0)
	<60	10(32.3)	11(35.4)	10(32.3)
Cigarette Smoking	Yes	8(17.0)	18(38.3)	21(44.7)
	No	19(43.2)	12(27.3)	13(29.5)
Drinking	Yes	8(18.6)	20(46.5)	15(34.9)
	No	19(39.6)	10(20.8)	19(39.6)
Location	Upper	2(15.4)	8(61.5)	3(23.1)
	Middle	9(23.7)	17(44.7)	12(31.6)
	Lower	16(40.0)	5(12.5)	19(47.5)
Histological differentiation	Well	0(0)	22(84.6)	4(15.4)
	Moderate	0(0)	6(24.0)	19(76.0)
	Poor	0(0)	2(15.4)	11(84.6)
Clinical stages	0	0(0)	9(100.0)	0(0)
	I	0(0)	20(95.2)	1(4.8)
	II	0(0)	1(6.2)	15(93.8)
	III/IV	0(0)	0(0)	18(100.0)

Table 2. Relationship between clinicopathologic characteristics and expression of NDRG2.

Variables		NDRG2 expression Positive (%)	Negative	P value
Gender	Male	51(69.9)	22	0.844
	Female	13(72.2)	5	
Age (years)	≥60	46(76.7)	14	0.066
	<60	18(58.1)	13	
Cigarette Smoking	Yes	35(70.0)	15	0.939
	No	29(70.7)	12	
Drinking	Yes	29(67.4)	14	0.568
	No	35(72.9)	13	
Location	Upper	11(84.6)	2	0.158
	Middle	24(63.2)	14	
	Lower	22(55.0)	18	
Histological differentiation	Well	20(76.9)	6	0.212
	Moderate	14(56.0)	11	
	Poor	10(76.9)	3	
Clinical stages	0/I	26(86.7)	4	0.006
	II	7(43.8)	9	
	III/IV	10(55.6)	8	

of NDRG2 and clinical stage in patients with ESCC. As the clinical stage progressed, the positive expression of NDRG2 gradually decreased.

Expression of NDRG2 by disease stage

Most of the positive granules of NDRG2 were located in cytoplasm and a few were in the nucleus, and no staining products were observed on the cell membrane; brown granules could be observed in the cytoplasm indicated by black arrows and nuclei indicated by red arrows of tissues adjacent to early ESCC, in mild-to-moderate dysplasia and in early ESCC, while only faint yellow staining could be detected in a large of advanced ESCC. The positive expression rate in advanced ESCC was significantly lower than in the other three tissue types (Figures 1 and 2).

Differential analysis of NDRG2 expression by disease stage

Positive expression rates of NDRG2 showed an obvious difference by disease stage, measuring 76.7% (23/30) in tissues adjacent to early ESCC, 74.1% (20/27) in tissues from mild-to-moderate dysplasia of esophageal squamous cells, 83.3% (25/30) in early ESCC, and 55.9% (19/34) in the advanced ESCC group ($p < 0.05$). The positive expression rate in tissues from advanced ESCC was significantly lower than in the other three tissue types, and the difference was statistically significant ($p < 0.05$) (Figure 3). In addition, individual IHC scores for NDRG2 expression in the four groups were 6.23 ± 1.91 points in tissues adjacent to early ESCC, 6.11 ± 2.53 points in tissues from mild-to-moderate dysplasia of esophageal squamous cells, 7.30 ± 2.26 points in early ESCC, and 4.88 ± 1.79 points in ESCC group, respectively. The individual IHC scores for NDRG2 expression in advanced ESCC were significantly lower than that of the other three tissues ($p < 0.05$).

Relationship of NDRG2 expression between early ESCC and advanced ESCC

In order to gain a deeper understanding of the role of NDRG2 in ESCC development, we analyzed the difference in NDRG2 expression between early ESCC and advanced ESCC on the basis of stratified clinicopathologic characteristics (Table 3).

Table 3 shows that there were significant differences in NDRG2 expression between early ESCC and advanced ESCC in men, patients aged <60 years, patients who consumed alcohol, and those with a medium or high disease grade. No significant differences in NDRG2 expression were seen in women, patients aged ≥60 years, or those with a low disease grade. Smoking habits and the ESCC location also did not influence NDRG2 expression.

Discussion

Human NDRG2 was first identified and reported by the Department of Biochemistry and Molecular Biology at the Fourth Military Medical University in China. It is identified with the gene bank login number of AF159092 [7–9]. NDRG2 is expressed in many human tissues, but especially in the brain, bones, liver, and heart [10]. Since its discovery, NDRG2 has attracted much attention as a candidate tumor-suppressor gene [11–13], as it is closely related to the proliferation, metastasis, and apoptosis of tumor cells [14,15]. Comparisons [4,16–20] of the expression of NDRG2 in ESCC, colorectal, liver, lung, and breast cancer and normal breast tissue have found that the expression level of NDRG2 is negatively correlated

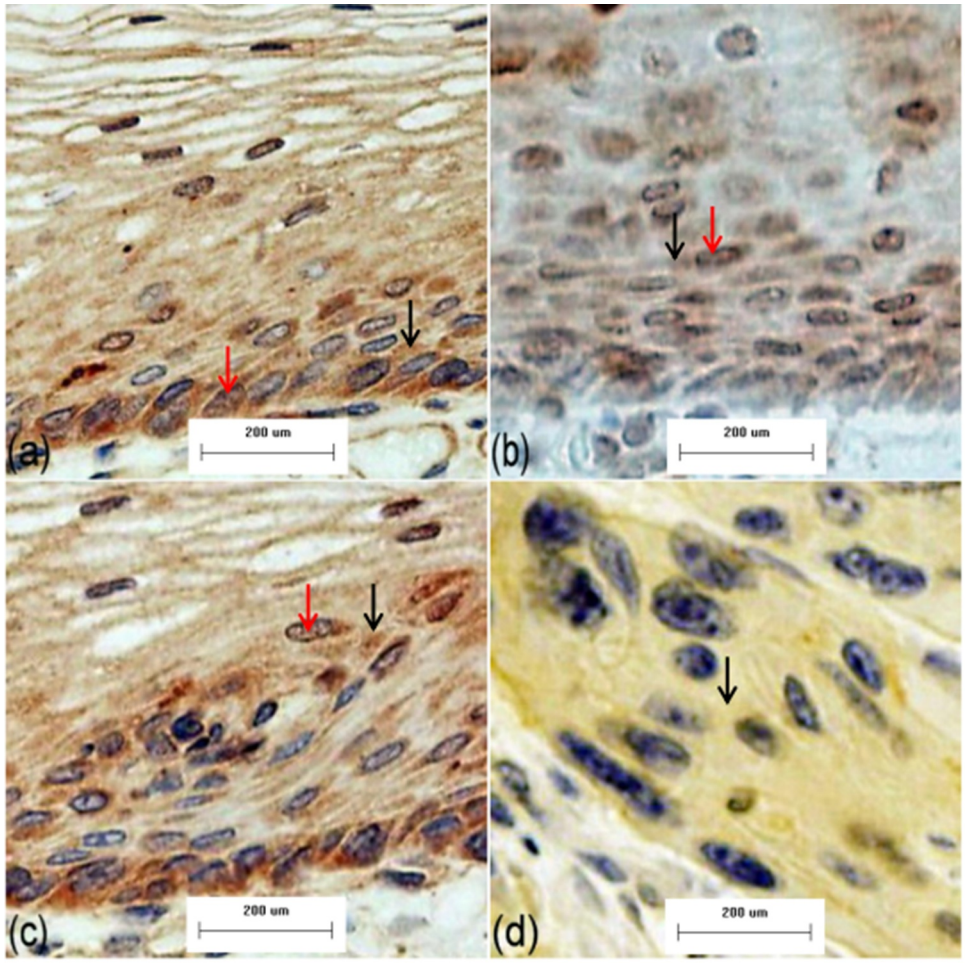

Figure 1. The location of NDRG2 expression in different stages of esophageal squamous cell carcinogenesis (×400, EnVision method, DAB staining): (a) tissues adjacent to early ESCC, (b) mild-to-moderate dysplasia of esophageal squamous cells, (c) early ESCC, (d) advanced ESCC. The black arrow indicates cytoplasmic staining, and the red arrow indicates nuclear staining. There are obvious nuclear staining in the tissues adjacent to early ESCC, suggesting nuclear translocation.

with the TNM staging and degree of differentiation of the tumor. Furthermore, levels are significantly lower in breast cancer tissue than in normal breast tissue.

The expression of tumor-suppressor genes is often lower in tumor tissue [21]. There are several reasons for this, the most common of which is a loss of heterozygosity at the genome level [22]. Other factors include promoter methylation [23], mutations in the gene encoding area or control area [24,25], and histone deacetylation [26,27]. The expression of tumor suppressor genes was different among different tumor types [28,29]. While the reduction in NDRG2 expression in advanced ESCC is probably mainly due to increased methylation of NDRG2 [30,31], it is still unclear whether the expression of NDRG2 is inhibited by other cytokines.

The positive expression rates of NDRG2 did not significantly vary between tissues adjacent to ESCC, mild-to-moderate dysplasia of esophageal squamous cell, and early ESCC. However, the positive expression rate of NDRG2 in advanced ESCC was significantly lower than in the other three tissues. This suggests that NDRG2 might be a tumor-specific marker and play a suppressing role in the early stages of ESCC. Furthermore, the level of NDRG2 expression helps to differentiate between early and advanced ESCC. Lower expression of NDRG2 may have important effects on the incidence and development of ESCC.

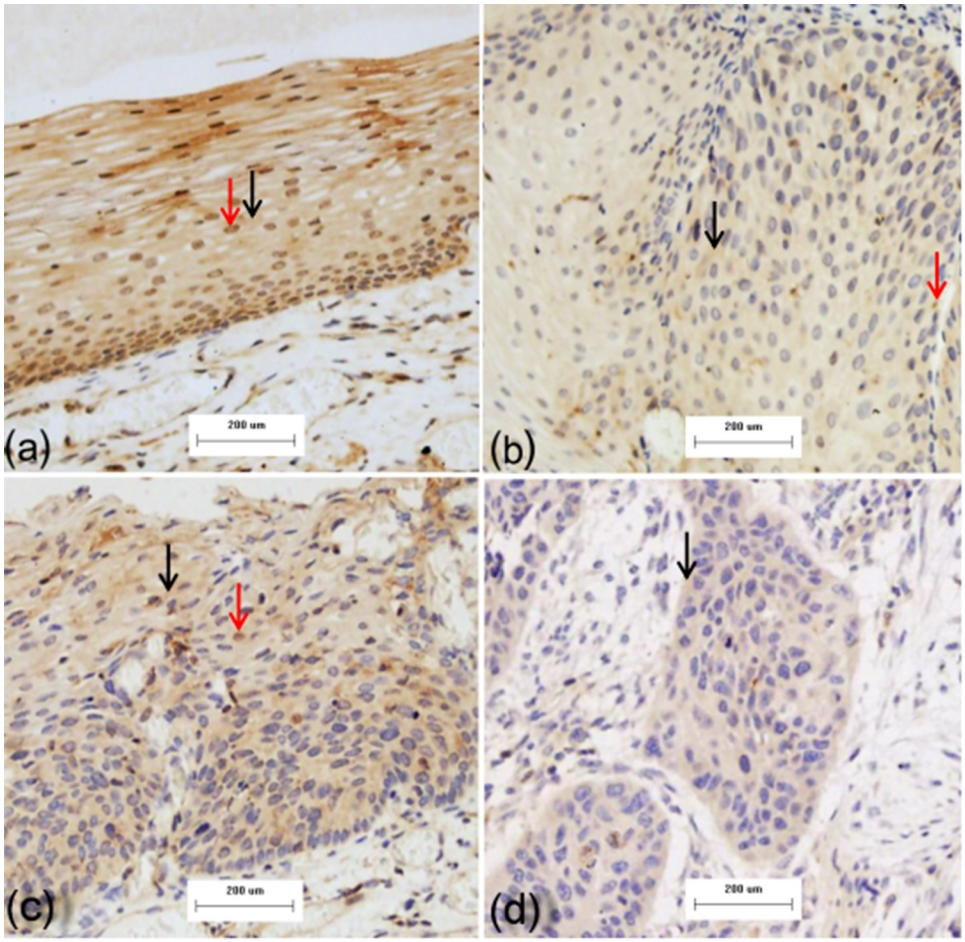

Figure 2. NDRG2 expression in different stages of esophageal squamous cell carcinogenesis (×200, EnVision method, DAB staining): (a) tissues adjacent to early ESCC, (b) mild-to-moderate dysplasia of esophageal squamous cell, (c) early ESCC, (d) advanced ESCC. The black arrow indicates cytoplasm staining and the red arrow indicates nucleus staining.

Further study may help not only to understand the mechanism of ESCC development but also to diagnose and treat ESCC.

Stratified analysis showed that there were significant differences in NDRG2 expression between early ESCC and advanced ESCC only in males, those aged <60 years, alcohol drinkers, and patients with a medium or high disease grade. No significant differences were seen in females, those aged ≥60 years, and patients with low-grade disease. Smoking habits and the ESCC location also had no influence. This suggests that gender, age, grading, and alcohol consumption affect NDRG2 expression in the progression of ESCC.

When all 91 patients were examined, no significant differences between NDRG2 expression and gender, age, drinking and smoking habits, and the location of esophageal lesions were found. Analysis restricted to the 64 patients with ESCC found no association with the degree of tissue differentiation. However, there was a significant difference and correlation between the expression of NDRG2 and clinical stage in this group. As the disease progressed, positive NDRG2 expression gradually decreased. There are various reasons why decreased NDRG2 expression might promote the progress of the tumor. Future research should concentrate on inhibiting the decrease of NDRG2 expression or improving its expression.

This study found that NDRG2 positive staining products were located only in the cytoplasm or nucleus; no staining products were observed on the cell membrane. Previous studies [32,33] show that, in the absence of stimulation, NDRG2 is located in the cytoplasm, but when cells are stimulated externally (by hypoxia, inflammatory factors, or heat shock), NDRG2 can transfer from the cytoplasm to the nucleus,

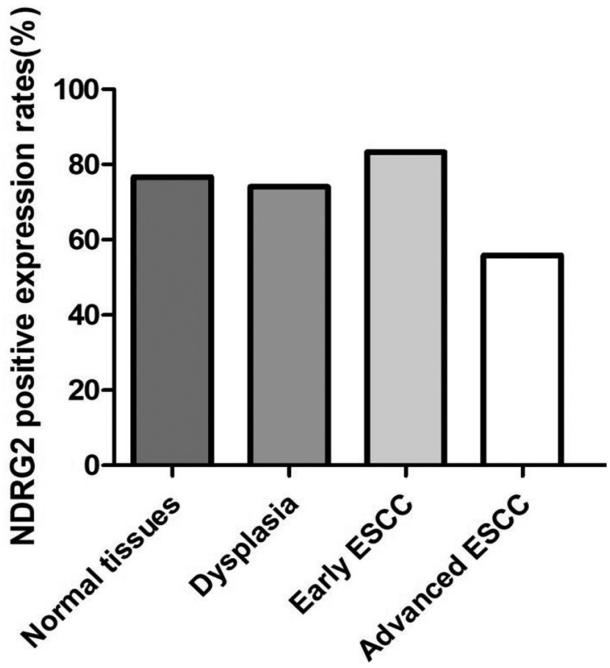

Figure 3. Different NDRG2 positive expression rates in four tissues. The positive expression rate in tissues from advanced ESCC was significantly lower than in the other three tissue types, and the difference was statistically significant ($p < 0.05$).

Table 3. Relationship of NDRG2 expression in different clinicopathologic characteristic subgroups between early ESCC and advanced ESCC.

Variable		NDRG2 expression				
		Early ESCC		Advanced ESCC		
Clinicopathologic characteristics		Positive(%)	Negative	Positive(%)	Negative	P value
Sex	Male	23(82.1)	5	13(48.1)	14	0.008
	Female	2(100)	0	6(85.7)	1	1.000
Age (years)	≥60	15(78.9)	4	16(66.7)	8	0.373
	<60	10(90.9)	1	3(30.0)	7	0.008
Smoking	Yes	16(80.0)	4	12(54.5)	10	0.081
	No	9(90.0)	1	7(58.3)	5	0.162
Drinking	Yes	16(80.0)	4	6(40.0)	9	0.032
	No	9(90.0)	1	13(68.4)	6	0.367
Location	Upper/middle	20(80.0)	5	10(66.7)	5	0.572
	Lower	5(100)	0	9(47.3)	10	0.053
Grading	High+medium	18(81.8)	4	10(43.5)	13	0.008
	Low	7(87.5)	1	8(72.7)	3	0.603

where it participates in a variety of cellular stress responses. In this study, it was found that both para-cancerous tissues and early ESCC showed significant staining in the nuclei, indicating that para-cancerous tissues differed from normal tissues and were likely in a state of stress. Many previous studies [34–38] have reported differences in the expression of these substances between para-cancerous and normal tissues, indicating that para-cancerous tissues may be clinically important.

One of the limitations was that this cross-sectional study with small sample size, especially for female, may weaken the generalizability of the results. For example, the positive expression rate of stage II patients was lower than that of stage III/IV patients. Another limitation was that fewer indicators were included in this study, excluding

treatment and prognosis. In the future studies, we will conduct prospective controlled trials with large sample sizes to verify the previous conclusions.

Conclusion

In conclusion, this study found that the expression of NDRG2 gradually decreased with the progression of esophageal lesions into advanced ESCC. This difference in positive expression rate was more obvious in male patients and patients under 60 years of age. Therefore, the detection of NDRG2 plays an important role in differentiating early ESCC from advanced ESCC.

Highlights

ESCC staging affects the expression of NDRG2 gene. The expression of NDRG2 in advanced ESCC was lower than that in other stages of ESCC. This difference in positive expression rate was more obvious in some patients.

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