

RESEARCH ARTICLE

Cytokeratin 15 is an Effective Indicator for Progression and Malignancy of Esophageal Squamous Cell Carcinomas

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Abstract

Purpose: To compare the expression level of CK 15 in normal esophageal and esophageal squamous-cell carcinoma (ESCC) tissues and analyse possible functions of CK15 in occurrence and development. **Materials and Methods:** Immunohistochemistry was used to compare CK14, CK15 and proliferating cell nuclear antigen (PCNA) expression levels in ESCCs. Expression level of CK15 was also assessed by Western blotting. In addition, levels of CK15, cytokeratin 19 fragment antigen 21-1 (CYFRA21-1) and PCNA were detected in serum by enzyme-linked immunosorbent assay (ELISA) and chemiluminescence methods. Relationships between clinicopathological parameters and CK14 and CK15 expression were then analyzed. **Results:** According to immunohistochemistry, in esophageal and intraepithelial neoplasia (SIN) tissues, the expression of CK14, CK15 and PCNA localized to basal layer of the epithelium. CK14 and CK15 levels were higher in normal esophageal squamous epithelial tissue than in SIN and ESCC, and greater in highly differentiated than poorly differentiated carcinoma tissue. By Western blotting, we found more pronounced expression of CK15 in normal esophageal tissue, compared with carcinoma tissue. The specificity of changed CK15 and CYFRA21-1 expression was respectively 90.0% and 96.7% in serum of ESCC patients. Joint detection could improve the sensitivity of esophageal carcinoma diagnosis. Relationships between CK14, CK15 expression and clinical parameters were not statistically significant ($P>0.05$). Postoperative survival in patients of CK14, CK15 positive expression was longer than with negative expression ($\chi^2=4.352, P=0.037$; $\chi^2=9.852, P=0.002$). **Conclusions:** CK15 expression decreased in esophageal squamous cell carcinoma tissue and serum of esophageal squamous carcinoma patients. We infer that CK15 may play an important role for the occurrence and development of esophageal squamous-cell carcinoma. In the future, CK15 may be used for the diagnosis, treatment and prognostic evaluation of esophageal squamous-cell carcinoma.

Keywords: Cytokeratin 14 - cytokeratin 15 - CYFRA21-1 - proliferating cell nuclear antigen - esophageal squamous

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Introduction

Human epithelium contains that comprise two types cytokeratin and every type have different subtypes. Epithelial tissues of different organ express different types of cytokeratin, with function of maintaining epithelial structure stability, participate in epithelial cell signal transduction, regulation of cell proliferation, apoptosis, malignant transformation and stress reaction (Moll, 2008). The interaction between each cytokeratin subtype can change cell function (Moll, 2008). Different carcinoma organizations exist specific type of cytokeratin expression, the expression of cytokeratin associated with the etiology of the tumor (Bragulla, 2009), apply in carcinoma diagnosis, classification and prognosis judgement.

In the subtypes of cytokeratin, CK5, CK14, CK15,

specifically express in the basal layer of the stratified epithelium. CK5, CK14 is thought to express by pairs, but CK15 has no obvious natural type II partner. In the human embryonic development stage, CK15 involved in the formation process of stratified epithelium tissue (Zhan, 2007), CK15 different expression may be related to distant metastasis, invasive ability in basal cell carcinomas and squamous-cell carcinomas. For a long time, CK15 can serve as a marker of epidermal stem cells (Liu, 2003). The study found that CK15 express in normal esophageal squamous epithelial cells (Porter, 2000). CK15 expressed situation in esophageal squamous-cell carcinoma is not very clear. In the study, we examine the CK15 expression in ESCC and normal esophageal tissue by different methods. Discuss the relationship between the expression of CK15, CK14 in ESCC, normal esophageal tissue and

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intraepithelial neoplasia (SIN), detect the expression of CK15 and CK19 in serum of esophageal squamous carcinoma patients.

Materials and Methods

Tissue specimen

Sample of ESCC, normal tissue and SIN, which were used in Immunohistochemistry collected from the first hospital of Shanxi Medical University in China, which contains 55 cases after surgical resection. The ESCC patients were 33 males and 22 females, aged 40-80 years. All 55 patients didn't receive preoperative Chemotherapy and Radiotherapy. Tissues were fixed by paraformaldehyde, embedded by paraffin. The 55 ESCC tissues consisted of 24 cases of well differentiated, 17 cases of moderately differentiated, 14 cases of poorly differentiated. Tissue of next to carcinoma consisted of 21 cases of SIN, 55 cases of normal tissue.

Samples of ESCC and normal tissue used in Western blot, and serum collected from the Endoscopy center of first hospital of Shanxi Medical University in China, which contains 30 cases. The cases consisted of ESCC tissues and para cancer normal tissues (The distances from cancerous tissue to normal biopsy place were 5-15cm, There are no cancer cells by pathological diagnosis). Tissues were frozen by liquid nitrogen; Serum samples from 30 ESCC patients and 30 normal persons. Venous blood was extracted, 3000 rpm, 10-minutes centrifugation. The supernatant was divided into three copies, stored by -80 Celsius.

The study authorized by the Shanxi medical university Ethics Committee in China.

Immunohistochemistry staining

Immunohistochemistry staining was performed using the streptavidin-peroxidase method. The primary antibodies in this study were rabbit anti CK14 (ZSGB-BIO Beijing China, 1: 100), Rabbit anti CK14 (BIOS, 1:200), Mouse anti PCNA (BOSTER, 1:100). The negative control, the primary antibodies were replaced by PBS. The sections were dewaxed in xylene and blocked endogenous peroxidase in 5% H₂O₂. The antigen retrieval for CK14 used citrate (PH6.0), at 120 Celsius, 3 minutes. The antigen retrieval for CK15 used pepsase, 10 minutes and The antigen retrieval for PCNA treated in EDTA (PH9. 0) at 120 Celsius, 3 minutes. Then all slides were used for incubated at 4 Celsius overnight. After three washed, add the second antibody. Substrate was done in Diaminobenzidine (DAB), make fresh 10ml DAB solution.

Enzyme-linked immunosorbent assay and Chemiluminescence

CK15, and PCNA were examined using Enzyme-linked immunosorbent assay (ELISA) kits (Meilian Biothch Inc., Shanghai, China). CYFRA21-1 was examined using mothed of Chemiluminescence (HYBIOME, Suzhou, China). Operations of ELISA and Chemiluminescence according to the manufacturer's instructions. In order to 450 nm wavelength measured the absorbance value of the

ELISA. EVERESYS A1800 series was used in method of Chemiluminescence.

Western blot

forty milligrams esophageal squamous carcinoma and normal tissues were prepared, proteins were extracted, protein determination by the Bicinchoninic acid (BCA) method. Absorbance of 256 nm measure by Enzyme standard instrument. Protein samples were loaded onto a 10% SDS polyacrylamide gel. Samples were transferred to a Cellulose acetate membrane. Primary antibodies of CK 15 (ZSGB-BIO Beijing China 1:700) were used for overnight incubation at 4 Celsius. Then HRP-conjugated goat anti-mouse antibodies (ZSGB-BIO Beijing China 1:10000) were used for 2 h at 4 Celsius. B-actin was used as a reference.

Survival analysis

We further analyzed the relationship between expression of CK14 and CK15 and survival of 55 patients. The follow-up visits were conducted for all 55 patients. Survival time calculated by the month. The interval of survival time from the date of surgery to the date of the deadline of follow-up visit (30/6/2013) or date of death. Finally, preparing a graph of survival time.

Statistical analyses

Immunohistochemistry stained expression levels in the samples of ESCC, normal tissue and SIN tissues were compared by using SNK test to identify significant differences. Chi-square test was performed for the comparison between cases of positive expression. Survival analyses of 55 patients were detected by Kaplan Meier analyses, Log-rank test was used to explain the relationship between expression of CK14/CK15 and survival. SPSS Statistics (version 13.0) were used to the statistical analyses.

Results

The expression of CK14 and CK15 has a relationship to Proliferation and differentiation degree of ESCC, The CK15 expression may have the function to predict differentiation degree of cancer tissue.

In this study. We tested the CK14 and CK15 expression in ESCC, which contain different differentiation group. We found that expression of CK14, CK15 are in the cytoplasm of ESCC. The expression of CK14, CK15 located in the nests of ESCC. Positive rate of CK14 expression and CK15 expression is respectively 63.6%, 72.7%. We detect the expression intensity of CK14, CK15 by Image-J software. The intensity level be analysis by gray. In different differentiation degrees, The mean gray value of CK14 expression was (181.30±6.17) in the well differentiated group, in moderately differentiated tissue was (189.25±6.03) and in poorly differentiated tissue was (205.65±3.21). The mean gray value of CK15 expression in well differentiated groups was (193.33±3.84), in moderately differentiated tissue was (197.18±2.47) and in poorly differentiated tissue was (207.72±4.18). Moreover, the higher for the gray value represent extent of antibody

Table 1. Gray Value Levels of CK14, CK15, PCNA Expression in Different Differentiated ESCC Groups

Group	n	CK14	n	CK15	n	PCNA
Well	21	181.30 ±6.17	19	193.33±3.84	18	191.69±6.19
Moderately	8	189.25±6.03*	6	197.18±2.47*	7	182.68±12.84*
Poorly	11	205.65±3.21*&	10	207.72±4.18*&	11	172.98±8.49*&
F		70.61		48.132		12.287
P		<0.001		<0.001		<0.001

Table 2. Enzyme-linked Immunosorbent Assay Expression Level Analysis of CK15, CK19 (CYFRA21-1), PCNA in ESCC and Normal Groups

Group	CK15	PCNA	CK19(CYFRA21-1)
ESCC	97.85±0.38	6.32±0.29	2.16±0.299
Normal	194.2±6.50	6.37±0.27	1.28±0.098
z	2.967	1.22	3.052
P	0.03	0.22	0.02

Table 3. Diagnostic Analysis for Joint Detection of CK15 and CK19 in ESCC Patients

Group	Joint detection		
	CK15	CK19(CYFRA21-1)	Combined Detection
Sensitivity	33.3	15%	60%
Specificity	90	96%	90%

Table 4. Relationship between CK14, CK15 Expression and Clinicopathological Parameters in ESCC Patients

Clinicopathol-ogical parameters	n	CK14		P	CK15		P	
		+	-		+	-		
Gender	male	33	25	8	0.537	20	13	0.567
	female	22	15	7		15	7	
Age	≥60`	36	27	9	0.602	25	11	0.218
	<60	19	13	6		10	9	
Tumor size	≥3cm	38	28	10	1	25	13	0.62
	<3cm	17	12	5		10	7	
TNM stage	I-II	32	24	8	0.655	20	12	0.836
	III-IV	23	16	7		15	8	
Differentiated	Well	24	21	3	0.016	19	5	0.012
	Moderately	17	8	9		6	11	
	Poorly	14	11	3		10	4	
Depth of invasion	Outside serosa	27	21	6	0.409	20	7	0.114
	Inside serosa	28	19	9		15	13	
Lymphatic metastasis	exist	20	12	8	0.109	12	8	0.672
	inexistence	35	28	7		23	12	

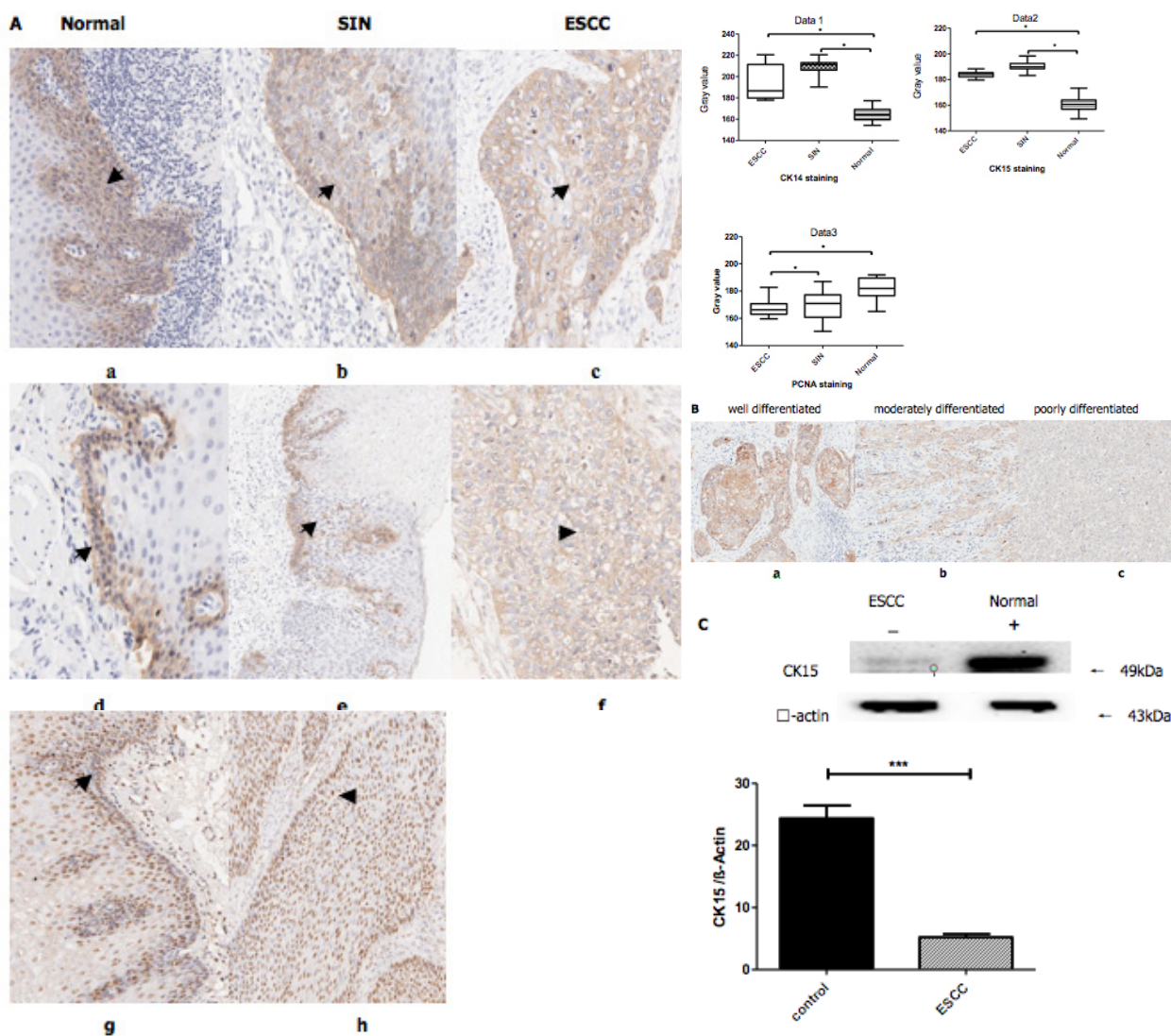
expression. This indicates that staining intensity of poorly differentiated tissue was significantly lower than the well differentiated group ($p<0.001$). To evaluate the proliferation level in ESCC. The immunohistochemical staining was performed. The expression of PCNA is in the nucleus, from well differentiated groups to poorly differentiated tissue, the expression was down regulated ($p<0.001$). In addition, the expression regions of CK14, CK15 and PCNA were same. The reasons illustrated CK14, CK15 participated the process of ESCC differentiation. (Table 1) To further evaluate the correlation between CK14, CK15 and PCNA expression. We used statistical analysis method of Pearson to perform the correlation analysis. Correlations were indicated between CK14 and CK15 (contingency coefficient, $C=0.725$, $P<0.05$). PCNA expression have the correlations with CK14 and CK15 ($C=0.585$, $P<0.05$ $C=0.405$, $P<0.05$). (Table 4)

The epithelial basal layer of CK14 and CK15 positive expression may has a relationship to the progression from normal tissue to the SIN and ESCC.

In the epithelial of other organ tissues, CK14 consistently as a marker of undifferentiated cells in the basal layers, they were regularly detected in the epithelial stem cells. We found, in normal esophageal tissue and

SIN epithelial, the positive staining cells of CK14 and CK15 were especially observed in the basal layer of epithelial. The positive expression local in the cytoplasm. Similarly, the PCNA express in epithelium basal layers. Positive rate of CK14, CK15, PCNA expressions are respectively 56.4%, 52.7%, 56.4%. Image-J software is used to measure the gray value. Respectively, compared with the expression between the ESCC, SIN and normal tissue, the staining intensity of CK14 and CK15 expression tended to up regulate in common tissue ($p<0.001$). We have observed contradictory result on PCNA. The staining intensity of PCNA expression tended to be down regulation ($p<0.001$). We explain the relationship between CK14, CK15 expression and clinicopathological parameters in 55 ESCC patients (The ESCC patients were 33 males and 22 females, aged 40-80 years), including age, sex, histological grade. Only histological grade associated with CK14, CK15 expression respectively. (Table 1)

We have checked the CK15 expressions in esophageal squamous-cell carcinoma tissues and normal esophageal tissues by western blot (The distances from cancerous tissue to normal biopsy place were 5-15cm, There are no cancer cells by pathological diagnosis). CK15 Protein distributed throughout the area which molecular weight is



49 kDa. Compared with tissue of esophageal squamous-cell carcinoma, CK15 expression level tended to be up regulation in normal tissue ($p<0.05$). (Table 3)

The analysis of the diagnostic value for CK15, CYFRA21-1 by Enzyme-linked immunosorbent assay and Chemiluminescence in human serum.

In order to evaluate the diagnostic value of CK15 and CK19 in ESCC patient. We detect the CK15 and CK19 expression in serum of ESCC patients. We have two

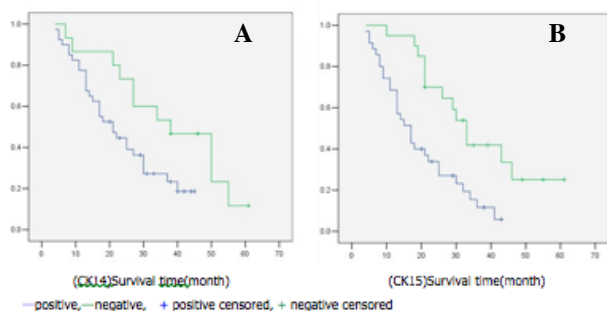


Figure 2. Kaplan-Meier Survival Analysis. A: CK14 negative positive ESCC cases. B: CK15 negative and positive ESCC cases

groups, esophagitis group and ESCC group. Compared with ESCC group, CK15 expression level tended to up regulated in serum of a normal group (diagnosed with esophagitis) ($p<0.05$). On the contrary, CYFRA21-1 expression level tended to down be regulated in the normal group ($p<0.05$). However, Compared with a normal group. The PCNA expression level didn't change in ESCC group ($p>0.05$). The specificity of the CK15 and CYFRA21-1 expressions were 90%, 96.7% respectively. The combined detection for CK15 and CYFRA21-1 can raise the sensitivity (60%) of diagnosis for esophageal squamous-cell carcinoma. (Table 2) So, we think that CK15 and CK19 can be used for the diagnosis of ESCC in the future.

Survival analysis

The CK14 and CK15 positive patients have the longer median survival time. To evaluate Whether CK14 or CK15 affects survival time of ESCC patients. We analyzed the relationship between expression of CK14 and CK15 and survival of 55 patients. Kaplan-Meier Survival curves were drawn. Median survival time of CK14 positive cases were

21.00±3.82 months. 95% confidence interval was (13.509, 28.491). Median survival time of CK14 negative cases were 38.00±8.08 months. 95% confidence interval was (22.163, 53.837). Median survival time of CK15 positive cases were 17.00±2.34 months. 95% confidence interval was (12.409, 21.591). Median survival time of CK14 negative cases were 33.00±2.67 months. 95% confidence interval was (27.774, 38.226). Compared with the negative cases, survival time of CK14 and CK15 positive cases tend to be down regulation ($\chi^2=4.352$, $P=0.037$, $\chi^2=9.852$, $P=0.002$). (Table 4)

Discussion

Cytokeratin is intermediate filament proteins, belong to one of the three elements of the cytoskeleton proteins. They are specially express in the epithelial cells. In the epithelium, the different cytokeratin expression is determined by the types of cells and differentiation degree of tissue. They are important to maintain cell shape, motility, the integrity of the structure. Cytokeratin is widely used as a marker in tumor pathology diagnosis and prognosis judgment of patient (Moll et al., 2008). At present, studies suggest that cytokeratin is associated to the generation of tumor, many cytokeratin subtypes can serve as a marker for stem cells.

CK15 belongs to the type I subtypes of cytokeratin, CK5, CK14 specifically express in epithelial cells. The CK15 is associated to the regeneration and development of epithelial tissue. During the stage of human embryonic development, CK15 involved in the formation process of stratified epithelium tissue (Leube et al., 1988). In the squamous epithelial tissue, CK15 is the basal specific keratin. In common neonatal human skin tissue, the expression of CK15 is continuous in the base layer (Pontiggia et al., 2009), and its expression is confined to the edge of the nests in squamous-cell cancer (Sakamoto et al., 2011). In the esophageal squamous epithelium, CK15 express in normal esophageal epithelium (Porter, 2000), CK15 is considered to be the marker of skin epithelial stem cells (Liu et al., 2003). We detected the expression of CK15 in ESCC and normal esophageal tissue by different methods. The study found that it may be associated with the origin and development for esophageal squamous cell carcinoma. In our study, we observed CK15 expression in normal tissue and SIN, the positively stained cells of CK15 were especially observed in the basal layer. The characteristics of epithelial tissue base layer decide the differentiation of epithelial tissue. It is associated with tumor stroma cells that is closely related to the tumor survival (Khanom et al., 2012). CK15 expression has changed in the skin and oral squamous-cell carcinoma and precancerous lesions (Khanom et al., 2012). In Immunohistochemistry staining, compared with ESCC and SIN, the staining intensity of CK15 expression tended to up regulation in normal tissue. Compared with esophageal squamous-cell carcinoma, CK15 expression level in normal tissue tended to be up regulation by Western blot. Compared with patients of esophageal squamous-cell carcinoma, CK15 expression level in serum from esophagitis persons tended to be up

regulation. At the same time, the expression of PCNA was jointly detected, CK15 and PCNA positive expressions were in the same area in normal esophageal squamous epithelial, SIN and ESCC. PCNA often as a symbol of the level of cell proliferation, and its expression is associated with the cell cycle, related to the replication of DNA and protein generated (Mailand N, 2013). There has been a controversy that CK15 could be used as a marker for the stem cell of epithelial, but this study showed that CK15 and PCNA were especially observed in the basal layer. We think that CK15 could be associated with stem cell in esophageal squamous epithelial. We propose that CK15 positive cells in basal layer involved in the process of normal esophageal squamous epithelial transformed into SIN and esophageal squamous-cell carcinomas. CK15 expression involved in the process differentiation of esophageal cancer.

Two types of cytokeratin which matching expression coordinately in the epithelial cell (Gu et al., 2007), but CK15 has no obvious natural type II partner (Fuchs et al., 1995). In normal oral squamous epithelium and oral squamous epithelium, after injury, CK14 gene was knocked out, CK15 expression correlated to the change of CK14 in protein level and gene transcription level (Waseem et al., 1999). In bronchial squamous epithelial cells, mRNA and protein about CK14 has changed in CK5/CK15 positive basal cells. In the study, we observed that positive expression of CK14, CK15 in the basal layer of normal tissue. In esophageal squamous-cell carcinoma, the positive expression are in the same area. The positive expression level down regulation. Correlation was indicated between CK14 and CK15 expression. The reasons contributed to analyze the interact for the function of CK14 and CK15. This result need the tests in future research.

CK19 is belonged to the type I keratin, its soluble pieces be released in the peripheral blood in the cancerous process (Takada et al., 1995). The soluble pieces of CK19 were called in Cytokeratin 19 fragment antigen 21-1 (CYFRA21-1) which can be used in diagnosis of squamous-cell carcinoma. The expression specificity of CYFRA21-1 in squamous-cell carcinoma is relatively high. It generally indicated the cell apoptosis. Now, CYFRA21-1 is considered as a marker of squamous carcinoma, but compared with the specificity, its sensitivity is poorer. According to the analysis of sensitivity and specificity, in the study, in serum of patients of esophageal squamous-cell carcinoma, the specificity of the expression of CK15 and CYFRA21-1 was 90%, 96.7% respectively. The Combined detection for CK15 and CYFRA21-1 can raise the sensitivity (60%). Joint detection of CK15 and CYFRA21-1 expression may improve the diagnosis of esophageal squamous-cell carcinoma.

The research of relationship between CK14, CK15 expression and clinic pathological parameters show that only histological grade associated with CK14, CK15 expression. Meanwhile, compared with ESCC and SIN, the staining intensity of CK14, CK15 expression tended to up regulation in normal tissue. CK14 and CK15 could be applied in the discovery of precancerous lesions and the pathological diagnosis. Compared to the negative cases,

survival of CK14 and CK15 positive cases tend to be down regulation, CK14 and CK15 could be used to assess the prognosis in the future.

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