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Research Article

Correlation of CTSD, P63, and Ki67 Expressions with Risk Assessment of Oesophageal Squamous-Cell Carcinoma

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Objective. Creatine kinase (CK), cathepsin D (CTSD), Ki67, and tumour protein 63 (p63) have been proven to participate in the growth of some cancers. However, available literature suggested paucity of data on their involvement in oesophageal squamous-cell carcinoma (ESCC) development. *Methods*. We ascertained the presence of CK, CTSD, Ki67, and p63 expressions in ESCC to demonstrate the association between differentiation of ESCC and expressions of the abovementioned proteins. We collected related information on 48 patients prior to their division into well and poor differentiation groups, which were analysed retrospectively. Positive rates of protein expression were evaluated via immunohistochemistry. The proteins that were expressed positively in all the cases were selected. Comparison of the proteins within two groups was done to analyse the correlation between tumour differentiation and their expression. *Results*. We observed that CTSD, p63, and Ki67 were significantly and highly expressed in poorly differentiated patients with ESCC. *Conclusions*. This finding may suggest that the proteins were involved in ESCC progression, which may evidentially serve as potential markers of early identification and risk assessment of ESCC.

1. Introduction

One of the malignant cancers which is most often diagnosed among individuals is cancer of the oesophagus (EC), wherein it is divided into oesophageal adenocarcinoma (EAC) and oesophageal squamous-cell carcinoma (ESCC) based on histological classification [1]. In developing countries, especially in China, ESCC is a more prevalent EC. ESCC is caused by many factors, which include drinking, smoking, infection, family history, hereditary, and so on [2]. In order to monitor the development of ESCC and improve its prognosis, identification of potential proteins involved in ESCC progression is very necessary.

Creatine kinase (CK) is located in cytoplasm and mitochondria with the potential to promote cancer cell division and inhibit its apoptosis [3, 4]. Since 1970s, CK has been considered for diagnosis of different cancers [5]. On the one hand, ATP at glycolytic sites can maintain homoeostasis by promoting the conversion of ATP to phosphocreatine (PCr) through cytosolic CK coupling with glycolytic pathway. On the other hand, reduction of CK activity could inhibit the glycolytic activity but enhance oxidative phosphorylation, thereby leading to alteration in energy status in cancer cells [6]. The gene of cathepsin D (CTSD) is located on chromosome 11p in region p15, amid being a member of A1 family of aspartyl proteases [7]. In humans, CTSD is composed of 412 amino acidic residues, wherein 20 amino acids are in pre-part and 44 amino acids are in pro-part [8]. When CTSD becomes mature, it can stimulate cytochrome c (CytC) in mitochondrion to regulate intrinsic apoptosis pathway, which also plays a crucial role in metabolism of cancer cells [9]. As a family member of p53 gene, tumour

protein 63 (p63) is considered as a transcriptional factor which is mapped to chromosome 3q [10]. Usually, it includes 2 splicing variants, which are TAp63 and ΔNp63. Other studies have reported that increased ΔNp63 could promote tumourigenesis by activating β-catenin signaling pathway, which could in turn reduce its phosphorylation and intranuclear accumulation [11]. However, the tumour suppressive activity of TAp63 has been reported, wherein a balance is likely to exist in different tissues [12, 13].

As a proliferative associated antigen in nucleus, chromosomal location of Ki67 at 10q25-ter has been established, which includes 15 exons and 14 introns. Low Ki67 expression was detected in G1 and S phases before it reached the peak in early mitosis, amid absence in G0 [14]. Function of Ki67 can be regulated by phosphorylation via serine and threonine, which can inhibit cell proliferation when it is downregulated [15]. The progression and suppression of cancer involves multiple proteins with increasing evidence indicating that CK, CTSD, p63, and Ki67 may participate in tumour development.

Studies have discussed functions of CK, CTSD, p63, and Ki67, which revealed their effect in the development of tumour, albeit few works investigating these proteins systematically in EC. In this retrospective observational study, variable expressions of the aforementioned proteins in different differentiation were to demonstrate their role in progression of ESCC and provide evidence for identifying potential treatment targets and risk assessment markers of the disease.

2. Materials and Methods

2.1. Recruitment of Patients and Collection of Specimens. We recruited 48 individuals that have been diagnosed with EC (36 males and 12 females). Evaluation of differentiation grades was accomplished by judging based on the World Health Organization (WHO) guidelines. Approval was given to this study by Ethics Committee at Jiangsu University, wherein performance of the experiments was based on Helsinki declaration guidelines.

2.2. Immunohistochemistry. After their fixation in formalin solution, specimens were paraffin embedded with the waxy substance sectioned into 4 μm thickness before mounting them on glass slides. All the sections were immersed in xylene for deparaffinisation before rehydration in graded ethanol. The sections were heated over medium-high heat for 6 minutes and moderate heat for 18 minutes before immersion in solution of citrate buffer (pH 6) in a microwave oven to repair antigen. At ambient temperature, we blocked the activity of endogenous peroxidase with H_2O_2 (3%) for 10 minutes. Later, we added primary antibodies before their placement at 4°C overnight after washing of sections in PBS. Afterwards, we washed the sections as described above prior to addition of signal enhancer and 20 minutes of incubation at 37°C. After rinsing with PBS, tissues mounted on the slides were covered with secondary antibodies before incubation for 20 minutes at 37°C. Next, DAB (Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd., 2040A0925) was used as chromogen after washing in PBS. Later, the slides were counterstained with haematoxylin, before immersion in graded ethanol and xylene to cause dehydration and hyalinisation, respectively. Using the score standard, we calculated the positive cell percentage to be <11% (1), 11–50% (2), 51–75% (3), and >75% (4). Meanwhile, the respective score standard of immunostaining showed 0, 1, 2, and 3 for no, weak, moderate, and strong staining. Afterwards, we calculated the eventual scores through multiplication of the extent score with intensity score. In addition, the final results were considered as positive if the score was more than 2. The detailed information of antibodies is shown in Table 1.

2.3. Statistical Analysis. Quantitative data were presented with number of cases and constituent ratio. Data analysis was accomplished with SPSS 26.0 statistical tool, wherein association of CK, CTSD, p63, and Ki67 expressions with differentiation of EC was investigated with *t*-test. Accepted level of significance was set at $P < 0.05$.

3. Results

Figure 1 displays the expression of CK, p63, CTSD, and Ki67 in ESCC patients with well and poor differentiation. In the oesophagus of ESCC patients, CK, p63, CTSD, and Ki67 were positively expressed. Furthermore, assessment of images showed that p63, CTSD, and Ki67 were highly expressed in poor differentiated tumour compared with well differentiated tumour. However, the difference in CK expression in both cases was not obvious.

3.1. Characteristics of Patients. We recruited 48 patients in our study and divided them into two groups, namely, well and poor differentiation. In the well differentiation group, 18 (75%) males and 6 (25%) females were recruited with 19 (79.1%) patients being beyond 60 years, wherein 50% of them exhibited alcoholic and smoking habit. Furthermore, retrospective survey showed that 6 (25%) patients were expressed as nm23 positive, while we observed p53 expression in 17 patients, with 75% and 83.3% of them positively expressing 5-Fu and CK18, respectively. Noticeably, CK, p63, CTSD, and Ki67 expressions were positive in all the patients. The specific characteristics of ESCC patients in well differentiation are presented in Table 2. In the poor differentiation group, the participants comprised of 18 males and 6 females with 21 of them beyond 60 years. Those that smoked were 8, while 6 of them demonstrated alcohol habit. Additionally, in this group, p53 was expressed positively in 75% of them, CD56 in 83.3%, CK18 in 38.1%, CD5/6 in 90%, and Syn in 63%. Interestingly, CK, CTSD, p63, and Ki67 were also found to be expressed positively in all poorly differentiated patients, aside p40. The detailed information on ESCC patients with poor differentiation is depicted in Table 3.

3.2. Association of Tumour Differentiation with Expressions of CK, CTSD, p63, and Ki67 in ESCC. Proteins that were expressed positively in both well and poorly differentiated

Table 1: Detailed information of antibodies.

Product name	Manufacturer	Lot.	Dilution
Creatine kinase (CK)	Chun Fai International Development Co. Ltd.	20201101020	1 : 200
p63	Chun Fai International Development Co. Ltd.	20210701030	1 : 200
Cathepsin D (CTSD)	Abcam (UK)	ab75852	1 : 200
Ki67	Chun Fai International Development Co. Ltd.	20210701080	1 : 200

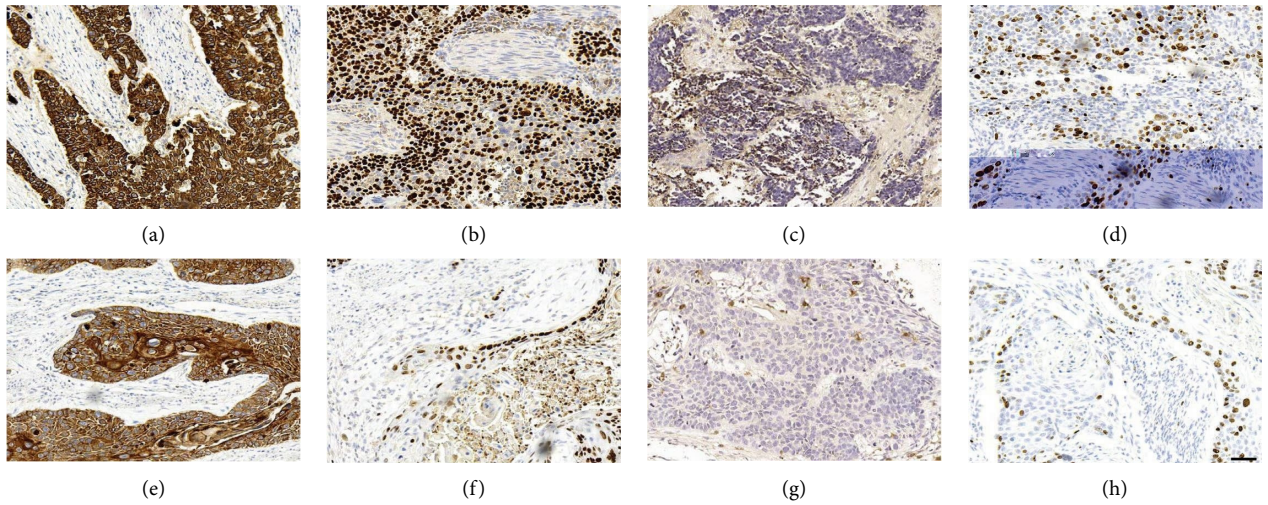

Figure 1: Immunostaining of creatinine kinase (CK), tumour protein 63 (p63), cathepsin D (CTSD), and Ki67 in oesophageal carcinoma (EC). (a–d) Expressions of CK, p63, CTSD, and Ki67 were analysed in the oesophagus of ESCC patients with poor differentiation, accordingly. (e–h) Positive staining of CK, p63, CTSD, and Ki67 in oesophagus tissues with well differentiated ESCC (200 magnification, bar = 50 μm).

patients were selected, amid comparison of their scores in the aforementioned subjects. Statistically, it was observed that CTSD expression correlated significantly with tumour differentiation ($P = 0.0021$), wherein the score was higher in ESCC patients with poor differentiation. Moreover, p63 and Ki67 expressions were significantly associated with tumour differentiation with the result also revealing that higher scores resulted in worsened tumour differentiation ($P < 0.0001$, $P = 0.0001$) as shown in Figure 2. However, the relationship between CK expression and tumour differentiation was not observed ($P = 0.2943$).

4. Discussion

Herein, we observed that CK, p63, CTSD, and Ki67 were expressed in both well and poorly differentiated patients with ESCC. In addition, the expressions of p63, Ki67, and CTSD were more likely to be higher in ESCC patients with poor differentiation compared with well differentiated ESCC patients.

The involvement of p63 in progression and suppression of tumours has been suggested, wherein it is divided into DNp63 and TAp63. It has been reported that DNp63 (as a transcriptional factor) could regulate DKK3 expression, which stimulated EC cell proliferation and was associated with bad prognosis of EC patients [16]. A study conducted by Albasri et al. to ascertain expression of p63 in CRC found substantial association of p63 expression with high Ki67 indexing, advanced stage of tumour, and poor prognosis [12, 17]. Indeed, some researchers have also demonstrated significant difference between expression of p63 and grading of other neoplasms like head and neck squamous-cell carcinoma and breast and lung cancers [18]. In contrast, Guo et al. discovered insignificant relationship among p63 expression and pathologic tumour stages as well as vascular invasion [19, 20]. Similar to most studies, we found that higher p63 expression resulted in poor differentiation in ESCC patients. Although TAp63 has been reported to inhibit tumour development, DNp63 isoform may often play a leading role. Thus, this may explain why prognostic studies are sometimes controversial. The CTSD has been reported as poor prognostic marker in breast cancer [21]. Moreover, excessive secretion of CTSD was discovered in the tumour microenvironment and various cancerous tissues [22]. This type of globular proteases caused invasion of cancer of lymph vessels, blood, and other tissues in tumours by attaching to proteins of other cell surfaces. According to previous studies, in urothelial bladder cancer patients, the activities of CTSD in their sera also proved to be directly proportional to the severity of cancer [23]. It often functioned as proteases which contribute to invasion of tumour cells [19]. In our study, CTSD was also considered as a marker of worsening condition of ESCC. The results showed that the ESCC patients with poor differentiation were more likely to have higher scores for

Table 2: Characteristics of patients with well differentiation in ESCC.

Patients' characteristics	Frequency (n)	Percentage
Sex		
Male	18	75
Female	6	25
Age		
≤60	19	79.2
>60	5	20.8
Smoking		
Yes	12	50.0
No	12	50.0
Alcohol		
Yes	12	50.0
No	12	50.0
CK expression		
Yes	24	100
p63 expression		
Yes	24	100
Ki67 expression		
Yes	24	100
nm23 expression		
Yes	6	25
No	18	75
p53 expression		
Yes	17	70.8
No	7	29.2
5-Fu expression		
Yes	18	75
No	6	25
CK18 expression		
Yes	20	83.3
No	4	16.7
CTSD expression		
Yes	24	100

Table 3: Characteristics of patients with poor differentiation in ESCC.

Patients' characteristics	Frequency (n)	Percentage
Sex		
Male	18	75
Female	6	25
Age		
≤60	21	87.5
>60	3	12.5
Smoking		
Yes	8	33.3
No	16	66.7
Alcohol		
Yes	6	25.0
No	18	75.0
CK expression		
Yes	24	100
p63 expression		
Yes	24	100
Ki67 expression		
Yes	24	100
p40 expression		
Yes	24	100

Table 3: Continued.

Patients' characteristics	Frequency (n)	Percentage
p53 expression		
Yes	18	75
No	6	25
CD56 expression		
Yes	3	20.0
No	12	80.0
CK18 expression		
Yes	8	38.1
No	13	61.9
CK5/6 expression		
Yes	9	90
No	1	10
Syn expression		
Yes	1	6.3
No	15	93.7
CTSD expression		
Yes	24	100

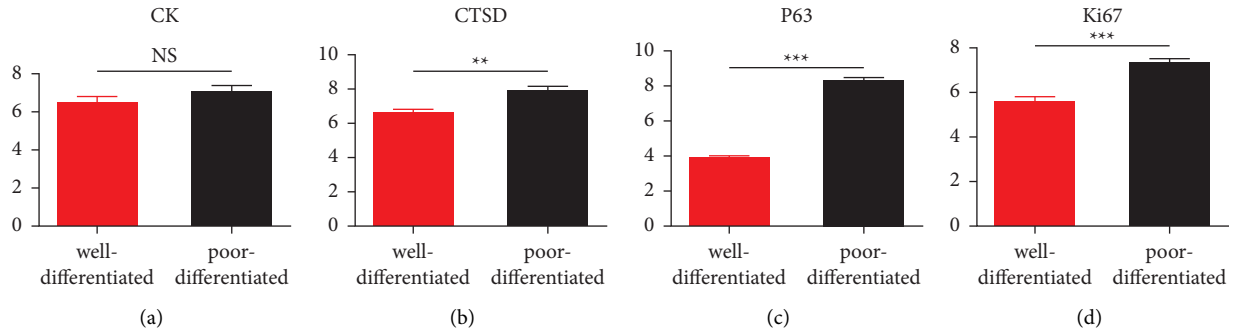

Figure 2: The difference in the expression of proteins in two groups (no significance (NS): $P > 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

CTSD expression. Similar to these results, Ketterer et al. showed that abrogation of CTSD expression in mammary epithelial cells could inhibit development of breast cancer significantly, thereby disturbing the dominant oncogenic signaling pathway (PI3K-mTOR pathway) [24]. Ki67 has been shown as a poor prognostic marker in many types of cancers, namely, oral squamous-cell carcinoma and breast cancer [25]. Some earlier studies in the United Kingdom discovered correlation of Ki67 proliferative biomarker with tumour grade in breast cancer [26]. Meanwhile, the significance of Ki67 in EC has not been reported yet. Notwithstanding, previous studies have shown Ki67 as a regulatory factor of cell proliferation via phosphorylation of serine and threonine [16]. Of note, relationship has been established between Ki67 expression and proliferation of intrinsic cell populations in various tumours [27]. Likewise, we observed that patients with positive Ki67 expression were more likely to show poor differentiation of ESCC. These findings implied that Ki67 is clinically and prognostically valuable as a reliable risk assessment marker.

In conclusion, these results suggest that higher expressions of CTSD, p63, and Ki67 were associated positively with ESCC differentiation. Given a high incidence of ESCC in China, our data suggest that these proteins may be applied for early identification and as a marker for risk assessment during diagnosis or treatment of ESCC. In addition, further designed studies like survival analysis are needed to confirm the value of these proteins in clinical and prognostic evaluation of ESCC.

Abbreviations

EC: Oesophageal carcinoma
EAC: Oesophageal adenocarcinoma
ESCC: Oesophageal squamous-cell carcinoma
CK: Creatine kinase
PCr: Phosphocreatine
CTSD: Cathepsin D
CytC: Cytochrome c
p63: Protein 63
WHO: World Health Organization.

Data Availability

The data that support the findings of this study are available upon reasonable request from the corresponding author.

Ethical Approval

Human experiments were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and all persons gave their informed consent prior to their inclusion in the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YC and HZ were responsible for conception and design, acquisition and analysis of data, and original draft preparation. QG and FR were responsible for acquisition, analysis, and interpretation of data. ZL and JZ were responsible for the creation of new software used in the work. MW and PH were responsible for conception and design and acquisition of data. JS was responsible for acquisition, analysis, and interpretation of data, original draft preparation, and revision. All authors read and approved the final manuscript.

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