Expression of Heat Shock Protein-27 (Hsp27) and P38MAPK in Esophageal Squamous Cell Carcinoma

ABDE 1 Yan Zhang*
ABEF 1 Zhiyin Feng*
C 2 Weina Wang
D 3 Juanjuan Dong
F 4 Xiaojin Gong
ADF 5 Hongwei Pu
AEF 1 Xiao Chen

* These authors contributed equally to this work: co-first authors Yan Zhang and Zhiyin Feng

Corresponding Authors:
- Xiao Chen, e-mail: xjchenxiao@sina.com;
- Hongwei Pu, e-mail: phwrose@sina.com

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Background:
Esophageal squamous cell carcinoma (ESCC) is a worldwide concern. This study looked at the relationship between the expression of differential proteins and the clinicopathological data and survival rate of ESCC patients to identify potential tumor markers for the growth and metastasis of ESCC.

Material/Methods:
This study included 162 patients who underwent surgical excision for management of ESCC. Fresh ESCC tissue and adjacent normal tissue specimens were collected. Protein expressions were detected by western blotting. The expression of Hsp27 and P38MAPK were detected by immunohistochemistry in formalin-fixed paraffin embedded primary tissue specimens.

Results:
The rate of positive Hsp27 and P38MAPK expression in ESCC tissue were higher than in normal esophageal tissue ($p<0.05$). The expression of P38MAPK was related to the depth of infiltration ($p<0.05$). The expression of Hsp27 was related to lymph node metastasis ($p<0.05$), but not with age, depth of infiltration, or tumor size. ROC were plotted to estimate the significance of the diagnosis: for Hsp27, AUC=0.735 ($p<0.05$), for P38MAPK, AUC=0.882 ($p<0.05$).

Conclusions:
The expression of Hsp27 and P38MAPK plays a role in ESCC development. Hsp27 and P38MAPK could be used as prognostic factors in ESCC.

MeSH Keywords: Esophageal Neoplasms • HSP27 Heat-Shock Proteins • Mitogen-Activated Protein Kinase 13

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Background

Esophageal squamous cell carcinoma (ESCC) has caused worldwide public health concern. The number of ESCC sufferers has increased gradually in recent years. The incidence rate varies in different physiographical regions, nations, and races. China has a high incidence of ESCC with a high mortality rate for ESCC patients. The Kazak population in northwest China has been reported to exhibit the highest incidence of ESCC. The mortality rate for esophageal cancer is 13.05 per 100,000 population in the Xinjiang region of China [1]. Zhang et al. [2,3] studied the differentially expressed proteins between human normal esophageal epithelial cells (NEEC) and ESCC cells; the differential protein expressions between NEEC and ESCC cells were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Heat shock protein-27 (Hsp27) is a member of a family of proteins. Their intracellular expression modulates the ability of cells to respond to several types of injury, heat shock, oxidative stress, and other unfavorable conditions. Besides its chaperone function, Hsp27 has also been shown to have an anti-apoptotic role by inhibition of caspase-dependent apoptosis, preventing a wide variety of apoptotic agents from causing cell death. Because over-expression of Hsp27 correlates with poor survival in ESCC patients, it may also be of importance.

The involvement of P38MAPK in cancer has been widely described. However, its role as an oncogene or tumor suppressor is unclear. Recently, numerous clinical trials have been initiated to use P38MAPK inhibitors in combination with chemotherapy for various types of cancer. As chemotherapy is a powerful inducer of P38MAPK activity, which in turn results in increased HAS2 activity and hyaluronic acid (HA) deposition, the use of either P38 or HA inhibitors could significantly dampen the cancer-promoting activity of the tumor microenvironment.

This study aimed to determine the expressions of Hsp27 and P38MAPK in ESCC by using western blotting and immunohistochemistry, and to statistically analyze the expressions of Hsp27 and P38MAPK, and determine the related clinicopathological characteristics of ESCC.

Material and Methods

Study design

Total protein of the ESCC tissue was evaluated by BCA protein assay (CWBio, Beijing, China). Then 30 μg of the ESCC tissue protein were analyzed by NuPAGE 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) that were transferred to polyvinylidene fluoride membranes (Invitrogen). The membranes were incubated overnight with polyclonal rabbit anti-Hsp27-antibody, polyclonal rabbit anti-P38MAPK-antibody (Bioss, Beijing, China), at 1: 1,000 dilution, respectively. The analysis was performed with NBT/BCIP staining. Protein expression was normalized to the quantity of beta-actin. The signal and grayscale values were visualized and analyzed by using ImageJ software, and grayscale value ratios were calculated. SP immunohistochemical method was used to detect the expression of Hsp27 and P38MAPK. The immunohistochemical EnVision-2 footwork method was used to detect the expression of Hsp27 and P38MAPK.

Immunohistochemical EnVision two footwork methods were used to determine the protein expression of Hsp27 and P38MAPK. The procedure was as follows: paraffin sections were dewaxed step by step, hydrated in water, incubated with 30 mL/L H2O2 (Bioss, Beijing, China) for 20 minutes at room temperature, and rinsed with PBS. The slices were placed in citrate buffer salt at pH 6.0; the repair antigen was added, and heated for 20 minutes in a microwave (temperature controlled at 95–100°C). The slices were then cooled to room temperature and rinsed with PBS. The antibody was then added and placed in the refrigerator at 4°C overnight. The slices were washed using the same method; DAB chromogenic reagent was added and the slices were placed in a wet box. Termination of the chromogenic reaction was achieved with tap water; then slice Wood grain redyeing, hydrochloric acid alcohol differentiation back to blue, and dehydration sealing. Western blotting was as follows: protease inhibitors (Bioss, Beijing, China) were added to cell lysates that were then placed on ice for 20 minutes. Lysates were then centrifuged at 14,000 rpm for 10
minutes at 4°C; protein (50 ug) was boiled for five minutes and separated by SDS-PAGE (Bioss, Beijing, China). Gels were transferred to a polyvinylidene fluoride PVDF membrane and blocked for one hour at room temperature. Blots were incubated with rabbit polyclonal antibody against PARD3 (Abcam, USA) or β-actin (Bioss, Beijing, China) at 4°C overnight, followed by incubation with the secondary antibody (Bioss, Beijing, China) at room temperature for one hour. A chemiluminescent substrate (CWBIO, Beijing, China) was added to visualize the specific bands. Quantity One software was used to quantify the intensity of each band and β-actin was used as the control.

Positive Hsp27 and P38MAPK expression was indicated by tan or brown granular particles in the cytoplasm. Expression in normal esophageal tissue was used as a control group.

Statistical analysis
Statistical analyses were conducted using SPSS17.0 software for analyzing sex, age, ethnicity, size of tumor, degree of differentiation, depth of infiltration, and lymphatic metastasis; we used the χ² test and t-test. Inspection level α=0.05, p<0.05 was considered statistically significant.

Results
Expression of Hsp27 and P38MAPK in ESCC and normal esophageal tissue by western blotting

Western blotting results showed that the grayscale value ratios of P38MAPK in the ESCC tissue (case 3) were 1.21, 1.41, and 0.98. The grayscale value ratios of P38MAPK in the normal tissue were 0.35, 0.25, and 0.19. These results showed that the expressions of P38MAPK had an increasing trend in the ESCC tissue compared with that of normal tissue (p<0.05) (Figure 1).

Expression of Hsp27 and P38MAPK in ESCC tissue by immunohistochemical method

Immunohistochemical results showed that the rate of expression of Hsp27 in esophageal cancer tissue compared to normal tissue was 71.0% (115/162) versus 24.0% (12/50) (Figures 2–5). The difference in rates was statistically significant (p<0.05) (Table 1).

Immunohistochemical results for the expression of P38MAPK in ESCC are shown in Figures 6–9. The rate of expression of P38MAPK in esophageal cancer tissue compared to normal tissue was 93.2% (151/162) versus 68.0% (34/50). The difference in rates was statistically significant (p<0.05) (Table 2).

Significance of the diagnosis by ROC curve analysis

According to the rate of expression of Hsp27 and P38MAPK in esophageal cancer tissue and normal tissue, ROC were plotted to estimate the significance of the diagnosis: Hsp27, AUC=0.735 (95.0% CI, 0.655–0.815), SE=0.04 with a significance level of p<0.001; and P38MAPK, AUC=0.882 (95.0% CI, 0.842–0.962), SE=0.03 with a significance level of p<0.001 (Figure 10).

Relationship between Hsp27 and P38MAPK expression and clinical pathological parameters

Hsp27 expression in patients without lymphatic metastasis and in patients with lymphatic metastasis was 62.6% and...
87.3%, respectively. The difference was statistically significant ($p<0.05$), which shows that Hsp27 expression was higher in patients with lymphatic metastasis. Hsp27 expression in ESCC was not correlated with sex, age, nationality, ethnicity, tumor size, or depth of infiltration. There was no significant difference ($p>0.05$) (Table 3).

P38MAPK expression in different depths of infiltration was 80.0%, 96.8%, 96.3%, and 88.0%, respectively. The difference was statistically significant ($p<0.05$). P38MAPK expression in Kazak ethnic patients and Han ethnic patients was 91.0% and 94.7%, respectively. There was no statistical significant difference ($p>0.05$). P38MAPK expression in patients without lymphatic metastasis and in those with lymphatic metastasis was 90.7% and 98.2%, respectively. Although P38MAPK expression was higher in patients without lymphatic metastasis, there was no statistical significant difference ($p>0.05$). These results indicate that P38MAPK expression in ESCC has no relationship with sex, age, ethnicity, differentiation degree, tumor size, or lymphatic metastasis ($p>0.05$) (Table 4).

**Table 1.** Hsp27 protein expression in esophageal cancer and normal esophageal tissues.

| Pathological characteristic | Total cases | Positive | Negative | Positive rate (%) | $\chi^2$ | $P$ value |
|-----------------------------|-------------|----------|----------|-------------------|---------|-----------|
| Cancerous tissue            | 162         | 115      | 47       | 71.0              | 35.120  | <0.01     |
| Normal tissue               | 50          | 12       | 38       | 24.0              |         |           |

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**Figure 2.** Positive expression of Hsp27 in esophageal squamous cell carcinoma (×100).

**Figure 4.** Negative expression of Hsp27 in esophageal squamous cell carcinoma (×100).

**Figure 3.** Positive expression of Hsp27 in esophageal squamous cell carcinoma (×400).

**Figure 5.** Negative expression of Hsp27 in esophageal squamous cell carcinoma (×400).
Heat shock protein 27 (Hsp27) is a member of the heat shock protein family which has been linked to tumor progression and, most interestingly, to chemotherapy resistance in cancer patients. There is evidence showing a role for Hsp27 in the balance between tumor dormancy and tumor progression, mediated by tumor-vascular interactions [4]. Hsp27 function is associated with deleterious outcomes in cancer and is associated with the development of drug resistance [5]. Some studies have found that exogenous Hsp27 uniquely blocks differentiation of monocytes to dendritic cells. This suggests that endogenous Hsp27 has immunoregulatory activities which could contribute to immunopathology. It has been speculated that Hsp27 enhances the ability of migration and invasion of cancer cells by preventing dendritic cell maturation [6]. Blocking the expression of Hsp27 protein has become an important research direction in tumor targeted therapy [7,8]. The over-expression of Hsp27 has been detected in invasive tumor cells in breast carcinoma, oral squamous cell carcinoma, and pancreatic carcinoma [9–11]. Some studies have also found that patients with upregulated expression of Hsp27 have better

| Pathological characteristic | Total cases | Positive | Negative | Positive rate (%) | $\chi^2$ | $P$ value |
|----------------------------|-------------|----------|----------|------------------|--------|-----------|
| Cancerous tissue           | 162         | 151      | 11       | 93.2             | 21.849 | <0.001    |
| Normal tissue              | 50          | 34       | 16       | 68.0             |        |           |

Table 2. P38MAPK protein expression in esophageal cancer and normal esophageal tissues.
long-term prognosis for carcinoma [12,13]. Lambot et al. [14] found Hsp27 protein expression increased drastically from dysplastic lesions to invasive carcinoma, being highest in the less differentiated areas.

In our study, we identified Hsp27 as an important participant in ESCC lymphatic metastasis. We found Hsp27 expression was significantly upregulated in ESCC tissues. Our data showed evidence of a significantly elevated level of Hsp27 in ESCC than adjacent tissue. Hsp27 expression in ESCC was shown to be associated with patients’ lymphatic metastasis. However, patient ethnicity had no effect on Hsp27 expression. These results indicate that Hsp27 plays a role in the carcinogenesis and development of ESCC. Further studies are needed to determine whether Hsp27 can be used as a pre-diagnosis indicator for ESCC; whether the expression of Hsp27 is closely related to drug resistance; and whether it can be used as a useful parameter to guide individual chemotherapy.

Mitogen-activated protein kinases (MAPKs) are involved in a variety of fundamental cellular processes, such as proliferation, differentiation, apoptosis, and survival. Previous studies have demonstrated activation of MAPKs pathways by pro-inflammatory cytokines in endothelial cells [15]. To the best of our knowledge, researchers have done very few studies on the relationship between P38MAPK and ESCC. P38MAPK (MAPK14) is a well-studied stress kinase that transmits numerous extracellular signals and is involved in multiple cellular processes.

**Table 3.** Correlation between Hsp27 expression and clinicopathological data.

| Characteristic                  | Negative | Positive | Positive rate (%) | t/χ²  | P value |
|--------------------------------|----------|----------|-------------------|-------|---------|
| Age (yr)                        |          |          |                   |       |         |
| Mean value                      | 64.77±9.07 | 62.92±8.96 | –                 | 1.184 | 0.238   |
| Sex                             |          |          |                   |       |         |
| Male                            | 29       | 79       | 73.1              | 0.734 | 0.463   |
| Female                          | 18       | 36       | 66.7              |       |         |
| Nationality                     |          |          |                   |       |         |
| Han                             | 30       | 65       | 68.4              | 0.735 | 0.482   |
| Kazakh                          | 17       | 50       | 74.6              |       |         |
| Differentiation degree          |          |          |                   |       |         |
| Moderate to high                | 35       | 88       | 71.5              | 0.077 | 0.840   |
| Low                             | 12       | 27       | 69.2              |       |         |
| Tumor size (cm)                 |          |          |                   | –     | 0.665   |
| Mean value                      | 2.82±1.88 | 3.03±1.85 | –                 | –0.665| 0.507   |
| Depth of infiltration           |          |          |                   |       |         |
| Mucosal & submucosa             | 8        | 12       | 60.0              | 1.338 | 0.294   |
| Muscular layer                  | 39       | 103      | 72.5              |       |         |
| Lymphatic metastasis            |          |          |                   |       |         |
| No                              | 40       | 67       | 62.6              | 10.723| 0.001   |
| Yes                             | 7        | 48       | 87.3              |       |         |

**Figure 10.** Receiver operating characteristic curve.

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The role of P38MAPK has been rigorously investigated in cancer. Originally proposed as a potential cancer suppressor [16], the role of P38MAPK in tumorigenesis, however, remains controversial. Recent studies of epithelial cells with disruption of P38MAPK have shown that its role in cancer cells is to suppress lung, liver, and colon tumor formation in vivo [17–19].

Over-expression of P38MAPK has been observed in hepatocellular carcinoma, lung adenocarcinoma, colorectal carcinoma, and head and neck squamous cell carcinoma [20–22]. In this study, we were able to establish a prognostic value for P38MAPK in human ESCC, where common P38MAPK over-expression correlated with advance tumor histology. The acquisition of migratory and invasive ability is a prerequisite for cancer cells to invade and metastasize. This process involves multiple intracellular cytoskeletal components and complex orchestration of biological pathways, often involving an intricate interplay between proteins. In the present study, we demonstrated that P38MAPK over-expression in ESCC was correlated with the depth of infiltration, suggesting that the detection of P38MAPK is valuable as a prognostic indicator of ESCC. However, other studies have reported conflicting results and more experiments are needed to confirm our findings. Identification of P38MAPK expression as the specific biomarker of ESCC could be an effective approach for determining the effect of treatment as well as the prognosis of tumors. At present, many trials have been initiated that use P38MAPK inhibitors in combination with chemotherapy for various types of cancer. In addition, hyaluronic acid (HA) is present as high and low molecular weight (HMW-HA, LMW-HA) polymers with LMW-HA generated by hyaluronidase-dependent fragmentation of HMW-HA. As chemotherapy is a powerful inducer of P38MAPK activity, the use of either P38 or HA inhibitors could significantly inhibit the cancer activity of the tumor microenvironment. From a therapeutic standpoint, the best approach to target HA-mediated tumor progression could be by blocking P38MAPK with pharmacological inhibitors, inhibiting HA synthesis targeting hyaluronidases, or disrupting HA-receptor interactions [23]. At present, fundamental research about effective methods of treatment of esophageal carcinoma is ongoing.

| Characteristic                     | Negative | Positive | Positive rate (%) | t/χ² | P value |
|-----------------------------------|----------|----------|-------------------|------|---------|
| **Age (yr)**                      |          |          |                   |      |         |
| Mean value                        | 65.45±4.45 | 63.31±9.05 |                   | 0.761 | 0.448   |
| **Sex**                           |          |          |                   |      |         |
| Male                              | 5        | 103      | 95.4              | 2.390 | 0.182   |
| Female                            | 6        | 48       | 88.9              |      |         |
| **Nationality**                   |          |          |                   |      |         |
| Han                               | 5        | 90       | 94.7              | 0.846 | 0.365   |
| Kazakh                            | 6        | 61       | 91.0              |      |         |
| **Differentiation degree**        |          |          |                   |      |         |
| Moderate to high                  | 10       | 113      | 91.9              | 1.449 | 0.463   |
| Low                               | 1        | 38       | 97.4              |      |         |
| **Tumor size (cm)**               |          |          |                   |      |         |
| Mean value                        | 2.14±1.12 | 3.03±1.89 |                   | −1.546 | 0.124   |
| **Depth of infiltration**         |          |          |                   |      |         |
| Submucosa                         | 4        | 16       | 80.0              | 4    | 0.16    |
| Muscular layer                    | 2        | 61       | 96.8              | 8.078 | 0.034   |
| Adventitia                        | 2        | 52       | 96.3              |      |         |
| Adjacent structure                | 3        | 22       | 88.0              |      |         |
| **Lymphatic metastasis**          |          |          |                   |      |         |
| No                                | 10       | 97       | 90.7              | 3.252 | 0.100   |
| Yes                               | 1        | 54       | 98.2              |      |         |

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In this study, we were able to establish a prognostic value for P38MAPK in human ESCC, where common P38MAPK over-expression correlated with advance tumor histology. The acquisition of migratory and invasive ability is a prerequisite for cancer cells to invade and metastasize. This process involves multiple intracellular cytoskeletal components and complex orchestration of biological pathways, often involving an intricate interplay between proteins. In the present study, we demonstrated that P38MAPK over-expression in ESCC was correlated with the depth of infiltration, suggesting that the detection of P38MAPK is valuable as a prognostic indicator of ESCC. However, other studies have reported conflicting results and more experiments are needed to confirm our findings. Identification of P38MAPK expression as the specific biomarker of ESCC could be an effective approach for determining the effect of treatment as well as the prognosis of tumors. At present, many trials have been initiated that use P38MAPK inhibitors in combination with chemotherapy for various types of cancer. In addition, hyaluronic acid (HA) is present as high and low molecular weight (HMW-HA, LMW-HA) polymers with LMW-HA generated by hyaluronidase-dependent fragmentation of HMW-HA. As chemotherapy is a powerful inducer of P38MAPK activity, the use of either P38 or HA inhibitors could significantly inhibit the cancer activity of the tumor microenvironment. From a therapeutic standpoint, the best approach to target HA-mediated tumor progression could be by blocking P38MAPK with pharmacological inhibitors, inhibiting HA synthesis targeting hyaluronidases, or disrupting HA-receptor interactions [23]. At present, fundamental research about effective methods of treatment of esophageal carcinoma is ongoing.
and there are still many pending disputes and question needing to solve [24–26]. Further studies are needed in this area. In our study, we were able to show the importance of P38MAPK and its effect on ESCC cell migration.

Conclusions

The expression levels of Hsp27 and P38MAPK play a role in the carcinogenesis and development of ESCC. Hsp27 and P38MAPK could be used as prognostic indicators in ESCC. Over-expression of Hsp27 was associated with lymphatic metastasis in patients with ESCC. Our experiment showed that P38MAPK was related to the high incidence of ESCC in depth of infiltration. Hsp27 and P38MAPK may play a role in the progression of esophageal cancer. Furthermore, we identified Hsp27 and P38MAPK as specific biomarkers of ESCC. Further studies are needed to confirm Hsp27 and P38MAPK as prognostic indicators for the effect of treatment and tumor prognosis.

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