Original Article

Novel prognostic value of nuclear lactate dehydrogenase-A in esophageal squamous cell carcinoma

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Abstract: Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors, and its prognosis is very poor. As a subunit of lactate dehydrogenase, LDH-A favors the conversion of pyruvate to lactate and is up-regulated in many tumors. It has been reported that LDH-A can promote cell growth and migration. However, the nuclear and cytoplasmic expression of LDH-A and the correlations and significance of this expression profile in ESCC have yet to be determined. In this study, the location of LDH-A protein in ESCC tissue from the histologically proven ESCC patients undergoing curative resection was determined by immunohistochemical staining. The nuclear and cytoplasmic expression of LDH-A was analyzed. Kaplan-Meier plots and the Cox proportional hazards regression model were used to analyze the prognostic value of nuclear and cytoplasmic LDH-A expression. The results showed that LDH-A protein in ESCC tissue was detected in both the nucleus and cytoplasm. High nuclear LDH-A expression was associated with malignant behavior, including tumor stage (P = 0.026) and lymph node metastasis (P = 0.022). High nuclear or cytoplasmic LDH-A expression alone was associated with lower overall survival (OS) rates (8.1%, P = 0.004 and 13.2%, P = 0.038, respectively). Moreover, in multivariate analysis, high nuclear LDH-A expression was the most significant factor indicating poor prognosis in ESCC patients (95% CI: 1.215–3.658, P = 0.008). In conclusion, our study showed that nuclear and cytoplasmic LDH-A expression correlate with more aggressive tumor behavior in ESCC and that nuclear LDH-A expression may serve as an effective prognostic marker in patients with surgically resected ESCC.

Keywords: Lactate dehydrogenase A, nuclear expression, esophageal squamous cell carcinoma, immunohistochemistry, prognosis

Introduction

Worldwide, the incidence and mortality of the two main types of esophageal carcinoma, squamous cell carcinoma and adenocarcinoma, remain high [1]. As the dominant type of esophageal cancer in China, the incidence of esophageal squamous cell carcinoma (ESCC) ranks sixth, while mortality due to ESCC ranks fourth. Moreover, these incidence and mortality rates have increased yearly from 2007 and 2008, respectively. Because the symptoms are not obvious at an early stage, ESCC patients are commonly diagnosed at an advanced stage; moreover, the lack of effective clinical methods for early detection and promising therapies together result in a poor prognosis [2, 3]. Therefore, prognostic biomarkers are needed to identify the subset of patients with a high risk of disease failure to guide individualized treatment.

Lactate dehydrogenase (LDH) is a 140-kDa tetrameric molecule composed of two different subunits: LDH-A and LDH-B. LDH-A is mainly located in the cytoplasm, catalyzing the posi-
Expression of nuclear LDH-A in ESCC

The purpose of this study was to evaluate the nuclear and cytoplasmic expression of LDH-A and to analyze the correlation between this expression pattern and the clinicopathological parameters of ESCC. Moreover, the influence of nuclear and cytoplasmic LDH-A expression on the overall survival (OS) of patients with ESCC was assessed.

Materials and methods

Patient samples and characteristics

Eighty-four patients with diagnosed and pathologically confirmed ESCC were evaluated in the Meizhou People’s Hospital of Guangdong Province. Demographic and clinicopathological data, including age, sex, histological grade, tumor size, depth of invasion, and clinical stage (TNM stage), were collected from patient medical records. All patients underwent curative surgery between June 2003 and June 2007, and none of them received radiotherapy or chemotherapy prior to curative surgery. Written informed consent was obtained from all ESCC patients, and this study was approved by the research Ethics Committee of Meizhou People’s Hospital.

Immunohistochemical staining

Slides (4 μm) from formalin-fixed, paraffin-embedded tissue samples were used for immunohistochemistry (IHC). Tissue sections were dried in an oven at 65°C for 60 min. After deparaffinization and hydration in buffer (water), epitope retrieval was performed by boiling the sections in citrate buffer solution (pH = 6.0) for 10 min. The slides were then placed in buffer for at least 20 minutes at room temperature and washed three times for 3 min each in PBS. The sections were then incubated in buffer for at least 20 minutes at room temperature and washed three times for 3 min each in PBS. The sections were then incubated for 2~3 h at room temperature with rabbit anti-human LDH-A antibody (1:400; CST) or PBS as a negative control. After three washes in PBS, the slides were incubated with goat anti-rabbit/mouse horseradish peroxidase polymer (Envision PO System; DAKO) for 30 min followed by incubation with 3,3’-diaminobenzidine as chromogen for 30~60 s. The sections were counterstained for 6 min with Mayer’s hematoxylin and permanently mounted. The staining pattern (cytoplas-
Expression of nuclear LDH-A in ESCC

Figure 1. Expression of nuclear and cytoplasmic LDH-A by immunohistochemical staining, showing five typical ESCC staining patterns. Representative ESCC tumor samples showing the expression of nuclear and cytoplasmic LDH-A. A. Dual negative expression of nuclear and cytoplasmic LDH-A. B. Dual low expression of nuclear and cytoplasmic LDH-A. C. High cytoplasmic and low nuclear expression of LDH-A. D. Low cytoplasmic and high nuclear expression of LDH-A. E. Dual high expression of nuclear and cytoplasmic LDH-A. Left column: H&E staining, 50×; middle column: IHC, 50×; right column: black box area from the middle column by IHC, 200×.
mic or nuclear) was recorded, and the expression was evaluated separately for the nucleus and cytoplasm.

**Statistical analysis**

The detailed procedure for LDH-A staining is shown in Tables 1 and 2. Negative and weak expressions were classified as low expression, while moderate and strong expression was classified as high expression. The data were analyzed with SPSS software version 19.0. The Chi-square test was used to analyze the association between LDH-A\textsubscript{nucl}/cyto expression and clinicopathological features. Kaplan-Meier survival curves were employed, and the statistics were evaluated using the log-rank method to compare overall survival rates, while Cox regression analysis was used for the multivariate analysis. For all statistical analyses, only \( P \) values < 0.05 were considered statistically significant, and all \( P \) values were two-sided.

### Results

**Clinical characteristics and expression of LDH-A in 84 ESCC patients**

Our study included 68 males and 16 females, and the gender ratio of male to female was 4.25:1. The median age was 55 years (range 41-75 years). The numbers of grade I, II and III ESCC cases were 13 (15.5%), 64 (76.2%) and 7 (8.3%), respectively. The median tumor size (maximum diameter) was 5 cm, and the cases were classified into two groups based on depth of invasion: muscular layer, 26 cases, 31.0%; and serosa layer, 58 cases, 69.0%. Tumor stage was distributed among cases as follows: I+II, 52 cases (61.9%); III+IV, 32 cases (38.1%).

LDH-A protein was detected in the nucleus and cytoplasm, as shown in Figure 1. The number of cases with low and high nuclear expression of LDH-A was 47 (56.0%) and 37 (44.0%), respectively, and the number of cases with low
and high cytoplasmic expression of LDH-A was 32 (38.1%) and 52 (61.9%), respectively.

Relationship between nuclear/cytoplasmic LDH-A expression and ESCC patients’ clinicopathological variables

Associations between nuclear/cytoplasmic expression in ESCC and clinicopathological variables were assessed, as displayed in Table 3. High expression of nuclear LDH-A was found to significantly correlate with male gender ($P = 0.005$), advanced tumor stage ($P = 0.026$) and more lymph nodes metastases ($P = 0.026$). In addition, high expression of cytoplasmic LDH-A was significantly correlated with male gender ($P = 0.025$) and older age ($P = 0.012$). No significant correlation was detected between nuclear/cytoplasmic LDH-A expression level and other clinicopathological variables ($P > 0.05$).

Relationships between clinicopathological variables, nuclear and cytoplasmic LDH-A expression, and overall survival by univariate analysis

The follow-up information of the 84 patients was collected within the range from 1 to 83 months after surgery. The mean and median OS time was 27.08 months and 16.00 months, respectively, and the 3-year OS rate was 16.9%.

Univariate analysis showed that the OS rate in patients with high expression of nuclear/cytoplasmic LDH-A was significantly lower than that in patients with low expression (Figure 2). As shown in Table 4, the mean/median survival time for patients with high expression was significantly lower than for those with low expression.
Expression of nuclear LDH-A in ESCC

Table 4. Univariate survival analysis of 84 ESCC patients by log-rank test

<table>
<thead>
<tr>
<th>Patients features</th>
<th>N</th>
<th>Mean ± SE (months)</th>
<th>Median ± SE (months)</th>
<th>3-year OS rate (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>25.70 ± 3.69</td>
<td>14.00 ± 2.40</td>
<td>15.7</td>
<td>0.239</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>29.33 ± 5.61</td>
<td>24.00 ± 4.27</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Age at surgery (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.843</td>
</tr>
<tr>
<td>≤ 55</td>
<td>43</td>
<td>26.55 ± 5.04</td>
<td>14.00 ± 2.15</td>
<td>16.9</td>
<td></td>
</tr>
<tr>
<td>&gt; 55</td>
<td>41</td>
<td>25.53 ± 3.89</td>
<td>20.00 ± 3.46</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.832</td>
</tr>
<tr>
<td>≤ 5</td>
<td>44</td>
<td>23.31 ± 2.91</td>
<td>19.00 ± 2.21</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>&gt; 5</td>
<td>40</td>
<td>28.29 ± 5.55</td>
<td>13.00 ± 1.34</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.431</td>
</tr>
<tr>
<td>Muscular layer</td>
<td>26</td>
<td>28.95 ± 6.19</td>
<td>19.00 ± 7.94</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td>Serosa layer</td>
<td>58</td>
<td>24.52 ± 3.42</td>
<td>14.00 ± 2.40</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.039</td>
</tr>
<tr>
<td>I+II</td>
<td>52</td>
<td>30.73 ± 4.47</td>
<td>20.00 ± 3.76</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>III+IV</td>
<td>32</td>
<td>19.54 ± 4.18</td>
<td>11.00 ± 1.43</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>LDHANuc expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Low expression</td>
<td>47</td>
<td>33.97 ± 5.00</td>
<td>22.00 ± 2.89</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>High expression</td>
<td>37</td>
<td>16.86 ± 2.82</td>
<td>11.00 ± 1.05</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>LDHACyto expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.038</td>
</tr>
<tr>
<td>Low expression</td>
<td>32</td>
<td>33.55 ± 6.23</td>
<td>24.00 ± 3.35</td>
<td>21.6</td>
<td></td>
</tr>
<tr>
<td>High expression</td>
<td>52</td>
<td>21.48 ± 3.28</td>
<td>13.00 ± 1.39</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>LDHANuc and LDHACyto expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Dual low expression</td>
<td>23</td>
<td>35.52 ± 7.37</td>
<td>24.00 ± 2.95</td>
<td>24.1</td>
<td></td>
</tr>
<tr>
<td>Single high expression</td>
<td>33</td>
<td>29.93 ± 4.86</td>
<td>19.00 ± 2.56</td>
<td>23.3</td>
<td>0.977*</td>
</tr>
<tr>
<td>Dual high expression</td>
<td>28</td>
<td>13.82 ± 2.55</td>
<td>10.00 ± 1.16</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

Bold values indicate statistically significant differences. *P value of overall survival between patients with high nuclear and low cytoplasmic LDH-A expression and low nuclear and high cytoplasmic LDH-A expression.

Table 5. Multivariate survival analysis of 84 ESCC patients by Cox regression

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (HR)</th>
<th>95% confidence interval of HR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage*</td>
<td>1.737</td>
<td>1.001–3.017</td>
<td>0.050</td>
</tr>
<tr>
<td>LDHANuc expression**</td>
<td>2.108</td>
<td>1.215–3.658</td>
<td>0.008</td>
</tr>
<tr>
<td>LDHACyto expression**</td>
<td>1.683</td>
<td>0.933–3.035</td>
<td>0.084</td>
</tr>
</tbody>
</table>

*Stages III+IV versus stages I+II; **High expression versus low expression.

Interestingly, when combining nuclear and cytoplasmic LDH-A expression, the OS rate of patients with dual high expression of nuclear and cytoplasmic LDH-A was substantially lower than that of patients with single high or dual low nuclear and cytoplasmic LDH-A (P < 0.001). Moreover, tumor stage also had a significant adverse impact on OS (P = 0.039).

**Prognostic significance of nuclear and cytoplasmic LDH-A expression in ESCC in multivariate analysis**

As shown in Table 5, multivariate analysis revealed that high expression of nuclear LDH-A...
Expression of nuclear LDH-A in ESCC

remained as the only independent prognostic factor for OS (hazard ratio (HR): 2.108, 95% confidence interval (CI): 1.215~3.658, \( P = 0.008 \)) compared with cytoplasmic LDH-A (HR: 1.683, 95% CI: 0.933~3.035, \( P = 0.084 \)) and tumor stage (HR: 1.737, 95% CI: 1.001~3.017, \( P = 0.050 \)).

Discussion

ESCC is the most common subtype of esophageal carcinoma, with an increasing incidence and high mortality in China [2]. Following continuous improvements in medicine in recent decades, diagnostic technologies and therapeutic strategies have greatly improved; however, the prognosis of ESCC remains poor, with a 5-year OS rate of approximately 10-41% [16, 17]. Therefore, it is crucial to identify a novel biomarker to predict the prognosis of patients and guide individualized treatment.

Malignant tumors are not only considered a genetic disease but also a metabolic disease, as alterations in glycolysis plays an important role in tumorigenesis [18]. LDH-A expression is mainly detected in the cytoplasm, and this enzyme catalyzes the positive reaction of the final step of glycolysis, converting pyruvate to lactate. Yao et al. showed that LDH-A promoted cell growth in the ESCC cell lines Eca109 and Caes17 via regulating the expression of CyclinD1, cleaved PARP and caspase 8, and knockdown of the expression of LDH-A was shown to inhibit tumorigenesis in vivo [13]. Indeed, inhibition of LDH-A expression suppresses tumor growth and metastasis and even causes the death of tumor cells [9, 19]. In this study, we found that 61.9% of ESCC patients had high expression of cytoplasmic LDH-A, which was correlated with poor survival. Many reports have demonstrated that total LDH-A is a potential prognostic marker in many tumors [10, 11], and our study also found that ESCC patients with high cytoplasmic LDH-A expression had a shorter mean/median survival time than those with low cytoplasmic LDH-A expression. In addition, high expression of cytoplasmic LDH-A was found to significantly correlate with male gender (\( P = 0.025 \)) and older age (\( P = 0.012 \)). In a similar finding, the expression of PKM2 was found to be much higher in ESCC than in normal tissue, and this expression is highly associated with many adverse clinical features and prognoses in ESCC, as reported by Zhan C et al. [7].

In addition to the cytoplasm, LDH-A has also been detected in mitochondria and nuclei. However, the role of nuclear LDH-A is still far from clear. The current study found that the expression level of LDH-A in the nucleus was high in 37 (44%) patients. Furthermore, significant correlations were detected between high expression of nuclear LDH-A and clinicopathological variables including advanced tumor stage (\( P = 0.026 \)) and positive lymph nodes metastasis (\( P = 0.022 \)), suggesting that LDH-A may play an important role in ESCC development. Nuclear LDH was found to localize to transcriptionally active regions of chromosomes [5, 20], and other studies have described nuclear LDH-A as mainly a single-stranded DNA-binding protein [21]. Together, these findings imply that nuclear LDH-A may have a similar function as other ssDNA-binding proteins, such as possible involvement in DNA transcription and/or replication and regulating the transcription of other genes, thereby regulating genes and proteins participating in tumor progression. Recently, Castonguay et al. reported that nuclear LDH can promote histone deacetylation by modulating the availability of NAD+, an essential ingredient for the activity of Sirtuin type 1 (SIRT1), which is intimately related to cell proliferation, differentiation, apoptosis, and metabolism [15]. Nuclear LDH-A may play a role in tumor progression by affecting histone deacetylation. Thus, it is not surprising that nuclear LDH-A expression showed good predictive value for the prognosis of ESCC patients in our univariate analysis.

High expression of LDH-A in the cytoplasm and nucleus appears to provide a metabolic and growth advantage to ESCC cells. Accordingly, patients with dual high expression of nuclear and cytoplasmic LDH-A showed the worst OS (\( P < 0.001 \)). Moreover, multivariate analysis showed that nuclear LDH-A expression had the greatest potential value in prognostic evaluation (HR = 2.108, \( P = 0.008 \)). Many studies have shown that total LDH-A overexpression and/or increased plasma LDH are important factors affecting the prognosis of cancer patients [22, 23], but few reports have focused on nuclear LDH-A expression. Our study is the first to examine both the nuclear and cytoplasmic expression of LDH-A in ESCC, and our results suggest that nuclear LDH-A may be even more important when evaluating ESCC progression. The subcellular localization of
LDH-A should be taken into account in risk estimation and treatment consideration for ESCC patients, and the underlying mechanism warrants further investigation.

Interestingly, we also found that LDH-A expression was higher in male than female patients in both the nucleus and cytoplasm ($P = 0.005; 0.025$). The association of LDH-A expression with gender may be attributable to hormone levels, which requires further study.

Taken together, the results of this study suggest that the expression of nuclear LDH-A may serve as a prognostic indicator for poor survival in ESCC patients.

Acknowledgements

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Disclosure of conflict of interest

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References

Expression of nuclear LDH-A in ESCC


