Original Article

Predictive and prognostic value of ACSL4 and GPX4 in patients with esophageal squamous cell carcinoma receiving post-operative radiotherapy

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Background: Although multimodality treatment, including chemoradiotherapy and surgery has significantly improved the prognosis of patients with esophageal squamous cell carcinoma (ESCC), a valid predictor is crucial for individualized treatment. As acyl-CoA synthetase long-chain family member 4 (ACSL4) and glutathione peroxidase 4 (GPX4) are key genes with radiation responses and constituents of the ferroptosis signaling pathway, the present study adopted ACSL4 and GPX4 protein expression to explore their predictive and prognostic value in patients with ESCC receiving adjuvant radiotherapy.

Methods: A total of 108 patients with thoracic ESCC who had undergone radical surgery and adjuvant radiotherapy were enrolled in the present retrospectively study. ACSL4 and GPX4 immunohistochemistry experiments were performed on paraffin-embedded tumor samples. The prognostic value of ACSL4 and GPX4 was examined using survival analysis, and the predictive value of ACSL4 and GPX4 for long-term survival was examined using univariate and multivariate Cox regression analyses, and verified by receiver operating characteristic (ROC) analysis.

Results: The survival analysis revealed that overall survival (OS) and disease-free survival (DFS) were significantly longer in the high ACSL4 expression group, and the DFS was significantly shorter in the high GPX4 expression group. The results of univariate and multivariate Cox regression analyses revealed that the ACSL4 expression level was an independent predictor for OS and DFS, and that the GPX4 expression level was an independent predictor for DFS. ROC analysis verified the predictive role of ACSL4 expression for DFS and OS, with an area under the curve (AUC) of 0.713 and 0.663.

Conclusions: The present study demonstrates that ACSL4 and GPX4 may serve as valuable prognostic biomarkers for long-term survival, and play a key translational role in individualized therapy for patients with ESCC.

Keywords: Esophageal squamous cell carcinoma (ESCC); acyl-CoA synthetase long-chain family member 4 (ACSL4); glutathione peroxidase 4 (GPX4); radiotherapy; prognostic value

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the second leading cause of cancer-related mortality worldwide, with an estimated 5-year overall survival (OS) rate of 22% (1). Radical surgery is the primary curative treatment for patients with ESCC, with local recurrence reported to be the major cause of failure. In recent years, multimodality treatment, including chemoradiotherapy and surgery has significantly improved the prognosis of patients with ESCC, and neoadjuvant chemoradiotherapy is widely recommended. In clinical practice, numerous patients with ESCC opt for receiving surgery first without neoadjuvant therapy, and adjuvant therapy may be crucial for such patients. Although post-operative radiotherapy for patients with ESCC is controversial, several retrospective and prospective studies have indicated that adjuvant radiotherapy significantly improved the survival of patients with locally advanced ESCC, compared with surgery alone (2-5). With the development of novel antitumor therapeutics, particularly immunotherapy and molecular targeted therapy, further improvements in the prognosis of patients with ESCC may be achievable. Therefore, strategies which can be used to select individuals with a poor prognosis for individualized treatment are critical. Valid predictors may aid in the selection of candidates for specific treatment and may lead to the development of more effective treatment strategies.

Ferroptosis, an iron-dependent and non-apoptotic form of regulated cell death with lipid peroxidation controlled by integrated oxidation and antioxidant systems, is associated with multiple pathologic diseases (6-8). Although the detailed mechanisms of ferroptosis remain obscure, an increase in the iron content can promote the growth of tumor cells, as iron is a key nutrient for cell proliferation and a co-factor for metabolic enzymes (9). Ferroptosis has been identified as a natural tumor suppressor mechanism in recent studies, and may be targeted for antitumor therapy. Research has indicated the complex ferroptosis-based crosstalk between cancer cells and immune cells (10). Furthermore, Yu *et al.* (11) found that the induction of ferroptosis enhanced the sensitivity to chemotherapy. The association between radiation and ferroptosis has raised concerns. Lei *et al.* (12) analyzed pre- and post-radiotherapy tumor samples, and found that radiotherapy induced lipid peroxidation and tumor-cell ferroptosis. Lei *et al.* (12) also indicated that ferroptosis is a main form of cell death in radiation-induced tumor suppression. Another study obtained a consistent conclusion, demonstrating that ferroptosis agonists and antagonists were associated with the efficacy of radiotherapy in tumor models (13). Acyl-CoA synthetase long-chain family member 4 (ACSL4) is a lipid metabolism enzyme required for ferroptosis (14-16). Glutathione peroxidase 4 (GPX4) mitigates lipid peroxidation and inhibits ferroptosis by utilizing reduced glutathione to convert lipid hydroperoxides to lipid alcohols (17,18). Available data indicate that ACSL4 promotes radiosensitization largely through the promotion of ferroptosis, and GPX4 promotes radioresistance largely through the inhibition of ferroptosis (12).

According to public databases, the clinical value of such ferroptosis-related genes has been rarely reported in patients with ESCC, particularly in the adjuvant radiotherapy setting. As ACSL4 and GPX4 are key genes with radiation responses and constituents of the ferroptosis signaling pathway, in the present study, ACSL4 and GPX4 protein expression was adopted to explore their predictive and prognostic value in patients with ESCC treated by adjuvant radiotherapy. We present this article in accordance with the REMARK reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1193/rc).

Highlight box

Key findings

- Acyl-CoA synthetase long-chain family member 4 (ACSL4) and glutathione peroxidase 4 (GPX4) may be biomarkers for the long-term survival of patients with esophageal squamous cell carcinoma (ESCC) receiving adjuvant radiotherapy.

What is known and what is new?

- ACSL4 and GPX4 are key genes with radiation responses and constituents of the ferroptosis signaling pathway.
- The present study adopted ACSL4 and GPX4 protein expression to explore their predictive and prognostic value in patients with ESCC receiving adjuvant radiotherapy.

What is the implication, and what should change now?

- They may help identify candidates for further treatment, and play a key translational role in individualized therapy for patients with ESCC.

Methods

Patients and treatment

From January, 2007 to September 2017, patients with locally advanced ESCC (T3-4 or N+) who had undergone radical

surgery and post-operative radiotherapy were reviewed. All patients received radical surgery including Ivor-Lewis or McKeown esophagectomy without neoadjuvant therapy. There were 70/108 (64.8%) of patients who received concurrent chemotherapy or sequential chemotherapy, and 38/108 (35.2%) of patients who did not receive post-operative chemotherapy due to old age (n=5), allergy to chemotherapeutic drugs (n=2), impaired liver function (n=3), bone marrow suppression (n=10) and patient refusal (n=18). Post-operative pathological tumor, node and metastasis (TNM) stage was decided according to the American Joint Committee on Cancer (AJCC) 8th staging system. Post-operative radiotherapy was performed within 12 weeks after surgical wound healing. The clinical target volume (CTV) was based on the position of tumor and positive lymph node, and the planning target volume (PTV) was generated by the 0.5 cm expansion around CTV. The prescription dose of intensity modulated radiation therapy was 50.4 Gy in 28 fractions. All the patients were consistently followed up every 3 to 6 months following adjuvant radiotherapy.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine (No. KY2020-032) and informed consent was taken from all the patients.

Data collection

This retrospective study was performed at Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. The final study population included 108 patients for analysis. Baseline information including age, sex, serum lactate dehydrogenase (LDH) levels, tumor length, tumor location, pathological grade, pathological T stage, pathological N stage and pathological TNM stage was derived from electronic medical records.

Immunohistochemistry staining

Immunohistochemical analysis was performed according to the protocol described in the study by Zong *et al.* (19). The expression levels of ACSL4 and GPX4 were examined on paraffin-embedded tumor samples obtained during surgery using rabbit anti-ACSL4 antibody (1:100; RRID: AB_2714020; Cat#ab155282, Abcam, UK) and anti-GPX4 antibody (1:100; RRID: AB_10973901; Cat#ab125066, Abcam, UK), respectively. The 4-μm-thick paraffin-embedded sections were deparaffinized and subjected to heat-mediated antigen retrieval using sodium citrate buffer (pH 6.0) for 10 min. The endogenous peroxidase activity was quenched using a 3% hydrogen peroxide solution and the slides were then blocked with 5% normal goat serum for 30 min. All the slides were incubated with anti-GPX4 or anti-ACSL4 antibodies at 4 ℃ overnight and then washed followed by incubation with goat anti-rabbit secondary antibodies at room temperature for 1 h. The same tissues without primary antibodies comprised the negative controls. The final score of ACSL4 and GPX4 was assessed by the staining intensity, which was subjectively assessed only in malignant epithelial cells according to a four-point scoring system comprising 0 (–), 1 (+), 2 (++) or 3 (+++). All the immunostaining results were examined and assessed independently on the collected patient samples by two pathologists who were blinded to the clinicopathological characteristics and clinical outcomes of the patients.

Statistical analysis

Disease-free survival (DFS) was calculated from the time of surgery to the first tumor recurrence or mortality from any cause. OS was calculated from the time of surgery to mortality from any cause. Survival curves were compared using the Kaplan-Meier method. Clinicopathologic characteristics, including age, sex, serum LDH levels, tumor length, tumor location, pathological grade, pathological T stage, pathological N stage and pathological TNM stage, were expressed as number and percentage. For the categorization of the continuous ACSL4 and GPX4 values into low and high expression groups, a cut-off point was selected for the measurements (range, 0–3), ≤1 was defined as low expression and >1 was defined as high expression. The representative immunohistochemistry images about the expression of ACSL4 and GPX4 proteins for 4 scoring points are shown in Figures S1,S2, respectively. The associations between the ACSL4 or GPX4 expression levels and the clinicopathologic characteristics of the patients were analyzed using a Chi-squared test or Fisher's exact test. Univariate and multivariate Cox regression analyses were performed to explore the predictive value of ACSL4 or GPX4 for OS or DFS. Receiver operating characteristic (ROC) analysis was adopted to verify the predictive ability. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated. Variables with a P value <0.10 in the univariate analysis were candidates for further multivariate analysis. A P value <0.05 was considered to indicate a

Table 1 Baseline characteristics

Characteristics	No. of patients (%)
Age (years)	
<65	73 (67.6)
≥65	35 (32.4)
Sex	
Female	19 (17.6)
Male	89 (82.4)
LDH (U/L)	
≤250	108 (100.0)
>250	0 (0.0)
Tumor length (cm)	
<5	59 (54.6)
≥5	49 (45.4)
Tumor location	
Upper	17 (15.7)
Middle	50 (46.3)
Lower	41 (38.0)
Pathological grade	
G1	11 (10.2)
G2	51 (47.2)
G3	31 (28.7)
Gx	15 (13.9)
Pathological T stage	
1	4 (3.7)
2	16 (14.8)
3	64 (59.3)
4	24 (22.2)
Pathological N stage	
0	37 (34.3)
1	47 (43.5)
2	18 (16.7)
3	6 (5.6)
Pathological TNM stage	
II	29 (26.9)
III	69 (63.9)
IVa	10 (9.3)
ACSL4	
Low	24 (22.2)
High	84 (77.8)
GPX4	
Low	18 (16.7)
High	90 (83.3)

LDH, lactate dehydrogenase; TNM, tumor, node and metastasis; ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase 4.

statistically significant difference.

Results

Baseline characteristics

Of the 108 patients in the final analysis, 89 (82.4%) were male and 19 (17.6%) were female. The mean age of the patients was 61.3 years (range, 44–80 years). A total of 17 (15.7%) patients were upper thoracic ESCC, 50 (46.3%) were middle thoracic ESCC and 41 (38.0%) were lower thoracic ESCC. All the patients had completed post-operative radiotherapy with a total dose of 50.4 Gy in 28 fractions. The patients were followed-up for a median time of 69.4 [interquartile range (IQR), 51.8–85.9] months, with a median OS of 33.4 (IQR, 15.2–65.3) months, median DFS of 22.3 (IQR, 9.9–58.4) months. The baseline characteristics of the included patients are summarized in *Table 1*.

Survival

At the final follow-up on December, 2021, a total of 70 (64.8%) patients experienced treatment failure or mortality, and the 1-, 2-, 3- and 5-year DFS rates were 65.7%, 46.3%, 36.1% and 24.1%, respectively. A total of 65 (60.2%) patients had succumbed by the final follow-up, including 58 patients from tumor progression, 2 patients from treatment-related side-effects, 5 patients from non-tumor-related diseases; 1-, 2-, 3- and 5-year OS rates were 86.1%, 59.3%, 44.4% and 26.9%, respectively. The survival analysis revealed that the DFS and OS rates were significantly longer in the high ACSL4 expression group (ACSL4 high *vs.* ACSL4 low, P<0.001; *Figure 1A*) (ACSL4 high *vs.* ACSL4 low, P<0.001;

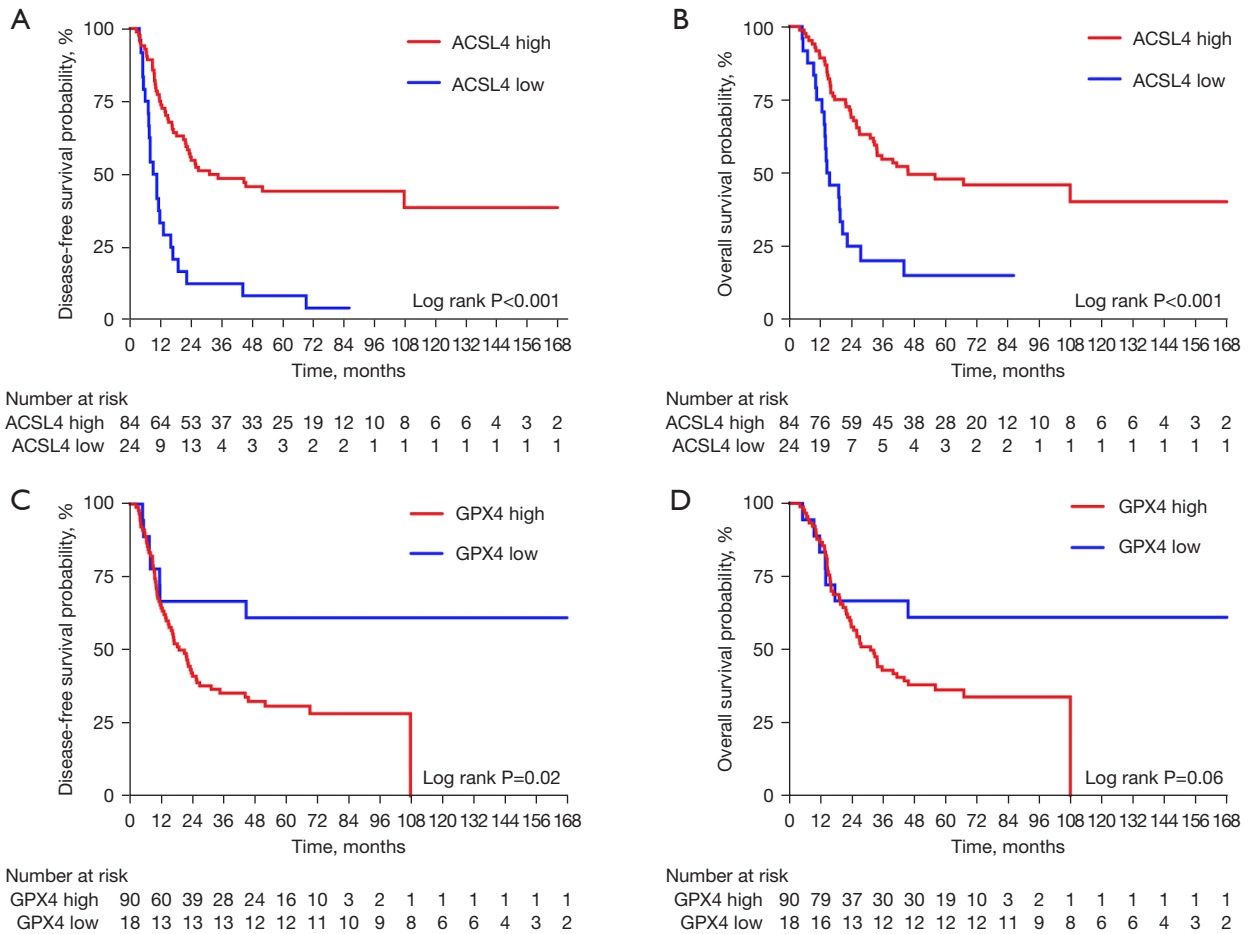

Figure 1 The survival analysis among different expression groups of ACSL4 and GPX4. (A) DFS curves for the high and low ACSL4 expression group. (B) OS curves for the high and low ACSL4 expression group. (C) DFS curves for the high and low GPX4 expression group. (D) OS curves for the high and low GPX4 expression group. ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase 4; DFS, disease-free survival; OS, overall survival.

Figure 1B). The DFS was significantly shorter in the high GPX4 expression group (GPX4 high *vs.* GPX4 low, P=0.02; *Figure 1C*); however, there was no significant difference in OS between the high and low GPX4 expression group (GPX4 high *vs.* GPX4 low, P=0.06; *Figure 1D*).

Univariate and multivariate cox regression analyses were performed to determine the clinicopathological risk factors associated with DFS and OS. ACSL4 expression, GPX4 expression, tumor length and pathological TNM stage were found to be independent clinical risk factors for DFS in the multivariate analysis (Table S1). ACSL4 expression, tumor length and pathological TNM stage were found to be independent clinical risk factors for OS in the multivariate analysis (Table S2).

ROC analysis verified the predictive role of ACSL4 expression for DFS and OS in the patients with ESCC. The area under the curve (AUC) of ACSL4 expression to predict OS was 0.663 (95% CI: 0.560–0.766) (*Figure 2A*), and the AUC of ACSL4 expression to predict DFS was 0.713 (95% CI: 0.616–0.811) (*Figure 2B*).

A multivariate logistic regression analysis was adopted to assess the predictive role of ACSL4 + GPX4 for OS or DFS. The AUC of ACSL4 + GPX4 to predict OS was 0.667 (95% CI: 0.565–0.769) (*Figure 3A*). The AUC of ACSL4 + GPX4 to predict DFS was 0.724 (95% CI: 0.628–0.819) (*Figure 3B*).

A multivariate logistic regression analysis including all the significant factors (ACSL4 expression, GPX4 expression, tumor length and pathological TNM stage) was adopted, whose AUC to predict OS was 0.756 (95% CI:

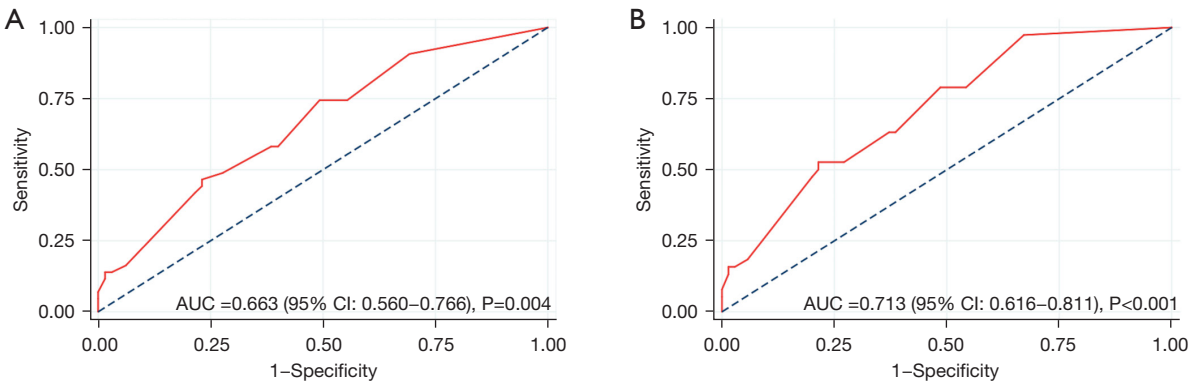

Figure 2 The ROC curves of ACSL4 expression to predict OS or DFS. (A) ROC curve to predict OS, with an AUC value of 0.663 (95% CI: 0.560–0.766). (B) ROC curve to predict DFS, with AUC value of 0.713 (95% CI: 0.616–0.811). AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic; ACSL4, acyl-CoA synthetase long-chain family member 4; DFS, disease-free survival; OS, overall survival.

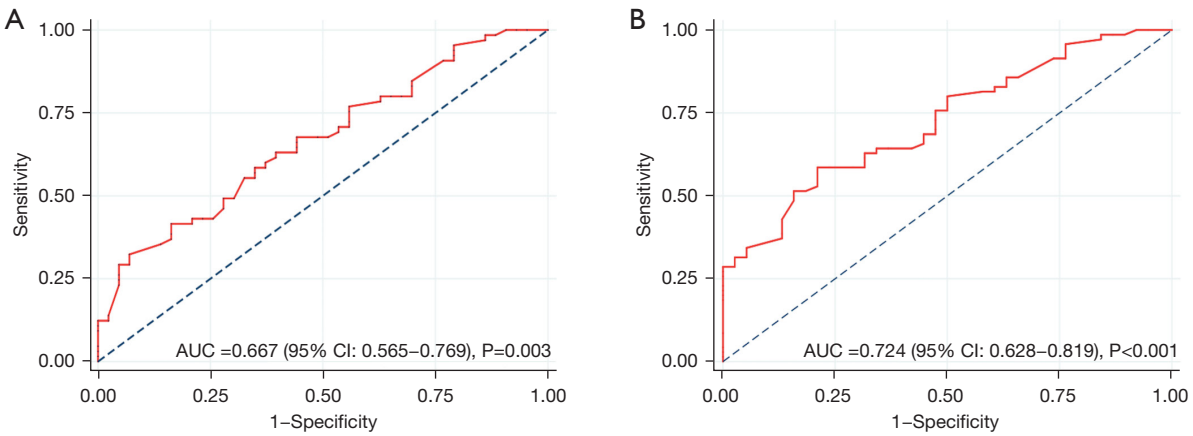

Figure 3 The ROC curves of ACSL4 + GPX4 to predict OS or DFS. (A) ROC curve to predict OS, with an AUC value of 0.667 (95% CI: 0.565–0.769). (B) ROC curve to predict DFS, with an AUC value of 0.724 (95% CI: 0.628–0.819). AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic; ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase 4; OS, overall survival; DFS, disease-free survival.

0.663–0.850) (*Figure 4A*), and the AUC to predict DFS was 0.778 (95% CI: 0.687–0.870) (*Figure 4B*).

Discussion

Ferroptosis, as a new form of regulated cell death, plays a dual role in tumorigenesis and antitumor therapy. Ferroptosis is an iron-dependent lethal mechanism which is considered a potential target for cancer therapy (20). The induction of ferroptosis not only inhibits tumor growth but also has the potential to enhance immunotherapy responses and overcome resistance to current cancer therapies (21). ACSL4 and GPX4 are vital regulators of ferroptosis, and several studies have revealed the value of ACSL4 and GPX4 serving as predictive and prognostic biomarkers in patients with tumors (22). In clinical practice, radiotherapy plays a crucial role in the treatment of ESCC; however, the prognostic value of ACSL4 and GPX4 in such a setting remains poorly defined.

To the best of our knowledge, this is the first study to explore the predictive and prognostic value of ACSL4 and GPX4 expression in patients with ESCC receiving adjuvant radiotherapy. The Kaplan-Meier survival analysis and multivariate Cox regression analysis revealed that a high

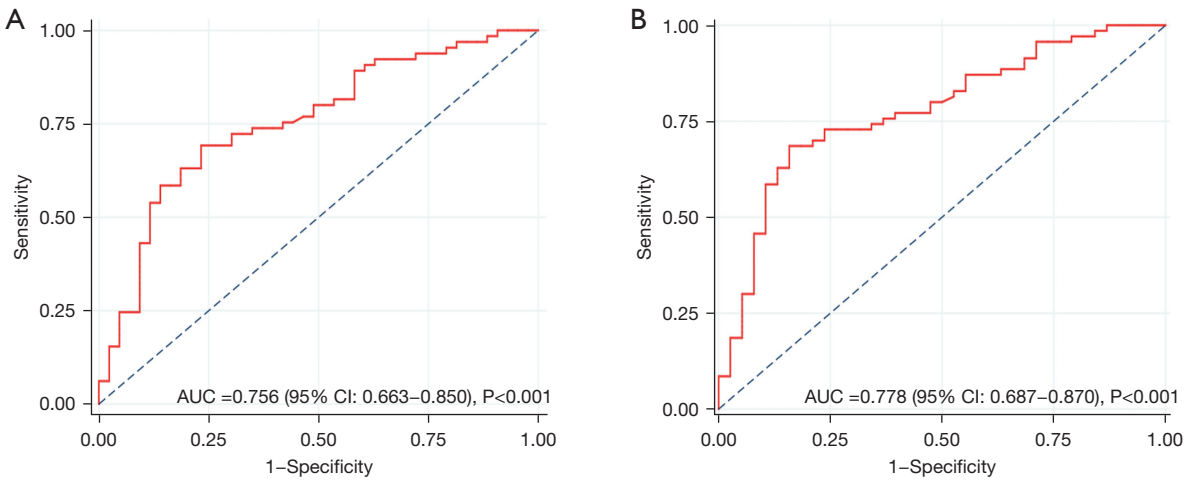

Figure 4 The ROC curves of all the significant factors (ACSL4 expression, GPX4 expression, tumor length and pathological TNM stage) to predict OS or DFS. (A) ROC curve to predict OS, with an AUC value of 0.756 (95% CI: 0.663–0.850). (B) ROC curve to predict DFS, with an AUC value of 0.778 (95% CI: 0.687–0.870). AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic; ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase 4; TNM, tumor, node and metastasis; OS, overall survival; DFS, disease-free survival.

ACSL4 expression was an indicator of a longer OS and DFS in patients with ESCC, and ROC analysis verified its predictive role. In addition, the present study indicated that a high GPX4 expression was a potential indicator of an unfavorable DFS in patients with ESCC, although the ROC analysis revealed a limited predictive value. The findings of the present study suggest that ACSL4 and GPX4 may function as biomarkers for the long-term survival of patients with ESCC receiving adjuvant radiotherapy, and may help to identify candidates for further treatment.

ACSL can catalyze fatty acids to form acyl-CoAs, thereby facilitating fatty acid metabolism and membrane modifications. A total of five isoforms of ACSL, including ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6, have different functions in fatty acid metabolism (23). ACSL4, as a sensitive monitor and a vital contributor of ferroptosis, is crucial for ferroptotic cancer cell death. Yuan *et al.* (16) found that ACSL4 protein expression was positively associated with the amount of ferroptosis in tumor cells, and ACSL4 promoted ferroptosis through the production of 5-hydroxyeicosatetraenoic acid. Previous studies have indicated that an elevated expression of ACSL4 is associated with $CD8^+$ T-cell infiltration, and improved immunotherapeutic responses in malignant cancers (24,25). Based on the currently available data, ACSL4 may play different roles in various tissues; the high expression of ACSL4 predicts the poor survival of patients with ovarian, liver and colorectal cancer, whereas the high expression of ACSL4 has been shown to be associated with improved outcomes of patients with brain, breast, bladder and lung cancer (23,25-28). However, the role of ACSL4 protein expression in patients with ESCC has not yet been systematically investigated. The present study revealed that ACSL4 plays a role in patients with ESCC receiving adjuvant radiotherapy.

GPXs belong to a family of phylogenetically related enzymes, and GPX4 is the only GPX that can catalyze the reduction of lipid peroxides in a complex cellular membrane environment (29). The inactivation of GPX4 in cancer cells with a mesenchymal state can lead to lipid peroxide accumulation and final ferroptosis, rendering these cancer cells highly dependent on GPX4 for survival (30,31). A high expression of GPX4 is regarded as a poor prognostic factor in patients with diffuse large B-cell lymphoma and lung adenocarcinoma (32,33). Shishido *et al.* (34) examined the expression of GPX4 in the pathological specimens of 97 patients with ESCC, and the prognostic analyses revealed that the upregulation of GPX4 was a poor prognostic factor. Shi *et al.* (35) found that GPX4 protein expression was significantly associated with the expression of immune cell markers in ESCC. According to the public results, GPX4 may be a key indicator of the prognosis of patients with ESCC; however, its prognostic value in patients with ESCC who have received post-operative radiotherapy has not been reported to date, at least to the best of our knowledge.

The present study revealed that GPX4 may play a role in patients with ESCC receiving adjuvant radiotherapy.

These results are consistent with the findings of previous studies. Shishido *et al.* (34) found that GPX4 positivity was significantly associated with tumor invasion depth, vascular and lymphatic involvement, lymph node metastasis, an advanced stage, a worse OS and recurrence-free survival in patients with ESCC who underwent radical esophagectomy. Tian *et al.* (36) observed a notable upregulation of ACSL4 expression and the downregulation of GPX4 expression following antitumor therapy, which suggested that ACSL4 and GPX4 protein expression may exert opposite effects on tumor progression.

ACSL4 and GPX4 may serve as valuable prognostic biomarkers and promising therapeutic targets, and exert key translational effects on the individualized therapy of patients with ESCC. In clinical practice, these patients with a poor prognosis may be selected as candidates for clinical trials for further treatment. Moreover, targeting ferroptosis is potentially a new avenue for antitumor therapy (37); it is possible to improve the prognosis of patients with ESCC by inducing ferroptosis through the promotion of ACSL4 or the inhibition of GPX4.

There were some limitations to the present study. First, the present study was based on retrospective data, and the sample size was relatively small, which may influence the reliability of the conclusions. Second, the present study focused on two ferroptosis-associated genes to evaluate their predictive and prognostic value in patients with ESCC receiving adjuvant radiotherapy; however, other genes which were not examined, may also participate this signaling pathway; thus, these remain to be explored. Third, The Cancer Genome Atlas dataset analysis and cell tests may be useful for further investigations into the potential roles of ACSL4 and GPX4 in ESCC. The authors aim to integrate more data to validate the current conclusions. In addition, the diverse methods of adjuvant chemotherapy employed in our study may impact the data credibility. We intend to conduct a retrospective, multicentre study to evaluate the roles of ACSL4 and GPX4 within a uniform treatment group and to validate the power of the predictive model. However, neoadjuvant chemoradiotherapy is more frequently utilized, and collecting data on postoperative radiotherapy will necessitate additional time.

Conclusions

The present study suggests that ACSL4 and GPX4 may be biomarkers for the long-term survival of patients with ESCC receiving adjuvant radiotherapy; they may also help identify candidates for further treatment.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1193/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1193/dss

Peer Review File: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1193/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1193/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine (No. KY2020-032) and informed consent was taken from all the patients.

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