High Expression of Methylenetetrahydrofolate Dehydrogenase 2 (MTHFD2) in Esophageal Squamous Cell Carcinoma and its Clinical Prognostic Significance

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Source of support: This study was partly supported by the National Natural Science Foundation of China (No. 81472329)

Background: Recently, targeted therapy for malignant tumors has developed rapidly, but there is still no effective targeted therapy for advanced esophageal squamous cell carcinoma (ESCC). Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) is a key enzyme involved in folate metabolism and is closely related to the proliferation in many cancers. However, few studies have explored the expression of MTHFD2 in ESCC and its prognostic significance.

Material/Methods: The expressions of MTHFD2, ki67, and p53 in ESCC tissues were detected by immunohistochemistry. Further, MTHFD2 expression level in ESCC and its correlations with patients' clinicopathological characteristics and survival prognosis were investigated.

Results: The enhanced expression of MTHFD2 was observed in ESCC specimens compared with adjacent normal tissue. The increased expression of MTHFD2 was closely related to pathological grading (P=0.020) and tumor TNM stage (P=0.019). In addition, patients with high expression of MTHFD2 had worse survival than those with low MTHFD2 expression (P<0.05). High expression of MTHFD2 in ESCC tissues was often associated with high expression of ki67 and p53 (P<0.05).

Conclusions: MTHFD2 had significantly enhanced expression in ESCC tissues and was associated with pathological grading and TNM stage. Taken together, high expression of MTHFD2 was an independent unfavorable prognostic parameter for overall survival (OS) of ESCC patients, suggesting that MTHFD2 might be a potential therapeutic target for ESCC in the future.

MeSH Keywords: Esophageal Neoplasms • Ki-67 Antigen • Methenyltetrahydrofolate Cyclohydrolase • Prognosis • Tumor Suppressor Protein p53

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/920259

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Background

Esophageal cancer ranks sixth worldwide in cancer-related deaths [1]. Recently, investigations showed that about 450 000 new patients were diagnosed with esophageal cancer each year, causing approximately 400 000 deaths worldwide [2]. Therefore, esophageal cancer is a disease with high incidence rates and poor prognosis. According to the published literature, China is a high-risk area for esophageal cancer, and the pathological type of most patients is esophageal squamous cell carcinoma (ESCC) [3]. In recent years, advances in molecular biology have improved cancer treatment and more and more targeted drugs have gradually begun to appear. The vast majority of targeted drugs have been exploited for treatment of tumors. However, drugs targeting ESCC still have limited efficacy. Novel therapeutic targets and prognostic biomarkers are urgently needed to improve the prognosis for patients with ESCC.

Methylenetetrahydrofolata dehydrogenase 2 (MTHFD2), also named methylenetrahydrofolata cyclohydrolase, was initially identified in Ehrlich ascites tumor cells in 1960 [4]. It is an essential enzyme in the mitochondria involved in cell activity and is encoded by genes on the human second chromosome [5]. MTHFD2 has a double function of formyltetrahydrofolate synthetase and cyclohydrolase [6], and it is necessary for enabling the cell to proliferate rapidly [7,8]. Recently, MTHFD2 has been shown to be highly expressed in liver cancer, breast cancer, and kidney cancer, and be associated with poor prognosis of patients [9–11]. The elevated expression of MTHFD2 has been proven to be closely related to the occurrence and development of tumors. However, there have been no relevant reports on the expression of MTHFD2 and its prognosis in ESCC.

First, we used a bioinformatics database to predict the expression of MTHFD2 in ESCC. Then, the expression levels of MTHFD2, Ki67, and P53 were detected by immunohistochemistry in ESCC tissues and the related normal tissues. Moreover, the chi-squared test was used to analyze the relationship between MTHFD2 expression and clinicopathological parameters. Kaplan-Meier univariate and Cox multivariate survival analysis were performed to evaluate the correlation between MTHFD2 expression and prognosis of ESCC patients.

Material and Methods

Bioinformatics analysis

To understand the feasibility of the experiment, the Oncomine database was used to predict the expression of MTHFD2 mRNA in ESCC in advance.

Patients and specimens

After approval by the Anhui Provincial Hospital and obtaining written informed consent from all subjects, research on the expression of MTHFD2 in ESCC was begun. A total of 78 histologically confirmed specimens of esophageal squamous cell carcinoma were collected from the Pathology Department of Anhui Provincial Hospital. These specimens were all from patients who underwent surgical resection of esophageal cancer in Anhui Provincial Hospital from January 2006 to October 2008. Follow-up lasted until July 2015. None of the patients received any treatment other than surgery, and the follow-up data were relatively complete. The clinicopathologic parameters of the patient included sex, age, pathological grading, lymph node metastasis, and TNM stage in patients. We obtained the TNM stage of patients and pathological grading of tissues according to the 7th edition of the Esophageal Cancer Staging System jointly formulated by the Union for International Cancer Control (UICC) and the American Joint Commission on Cancer (AJCC). Among these 78 patients, cases 1–61 of ESCC pathological specimens had corresponding normal tissues, and cases 62–78 were only ESCC pathological specimens.

Immunohistochemistry

First, HE staining was performed to distinguish cancerous and adjacent noncancerous tissue specimens. Then, immunohistochemistry was applied to examine the expression levels of MTHFD2. Conventional paraffin sections were dewaxed with xylene and dehydrated with gradient ethanol. Afterwards, the sections were washed with phosphate-buffered saline (PBS) and incubated at 37°C and 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. After antigen retrieval with citrate buffer (pH 6.0), sections were blocked in 10% sheep serum for 10 min. The sections were incubated with anti-MTHFD2 antibody (1: 500, Abcam, UK) at 4°C overnight, and then incubated with biotin-labeled antibody (Dako, USA) for 30 min at room temperature. Finally, the sections were colored by 3,3-diaminobenzidine (DAB) and stained with hematoxylin. PBS was used as negative control instead of primary antibody. Pathological specimens were assessed for expression of ki67 and p53 by immunohistochemistry.

Determination of immunohistochemical results

Two pathologists independently evaluated the immunohistochemical results of all sections using a double-blind method. We evaluated the positive expression of the gene from the 2 aspects of staining intensity and the proportion of positive cells. The standards for staining intensity were as follows: 0 points (no staining); 1 point (light brown), 2 points (yellowish brown), and 3 points (dark brown) (Figure 1). The percentage of positive tumor cells was classified as follows:
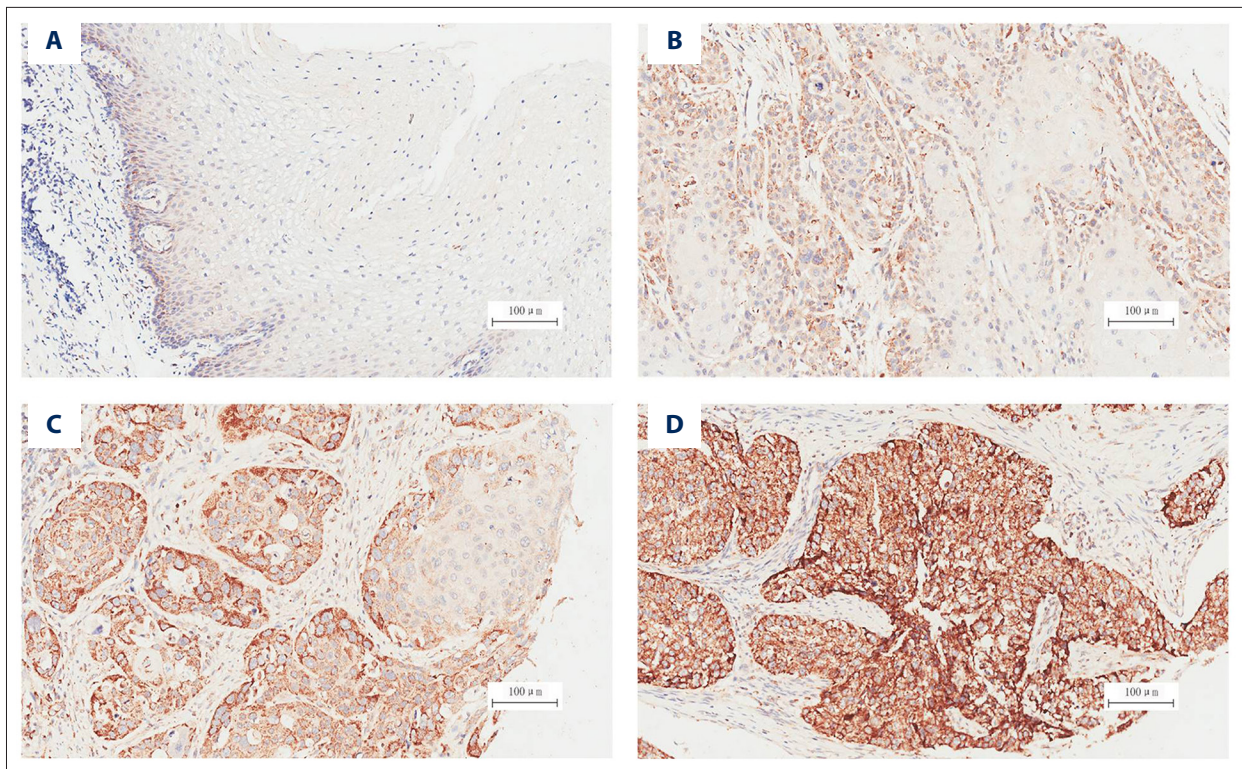
Figure 1. Immunohistochemical staining of MTHFD2 in esophageal squamous cell carcinoma (ESCC) tissues: (**A**) 0 points (no staining); (**B**) 1 point (light brown); (**C**) 2 points (yellowish brown); (**D**) 3 points (dark brown).
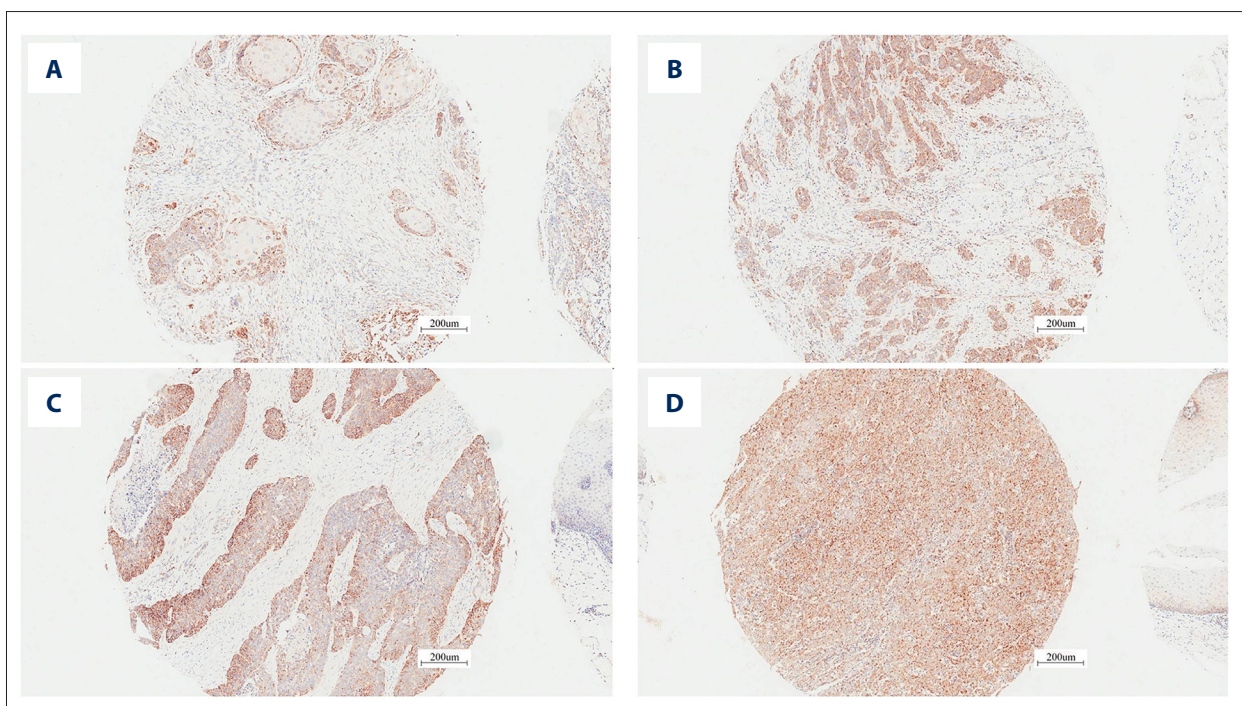

Figure 2. The percentage of positive tumor cells was classified as follows: (**A**) 1 point (1–25% of the positive cells); (**B**) 2 points (26–50% of the positive cells); (**C**) 3 points (51–75% of the positive cells); (**D**) 4 points (76–100% of the positive cells).
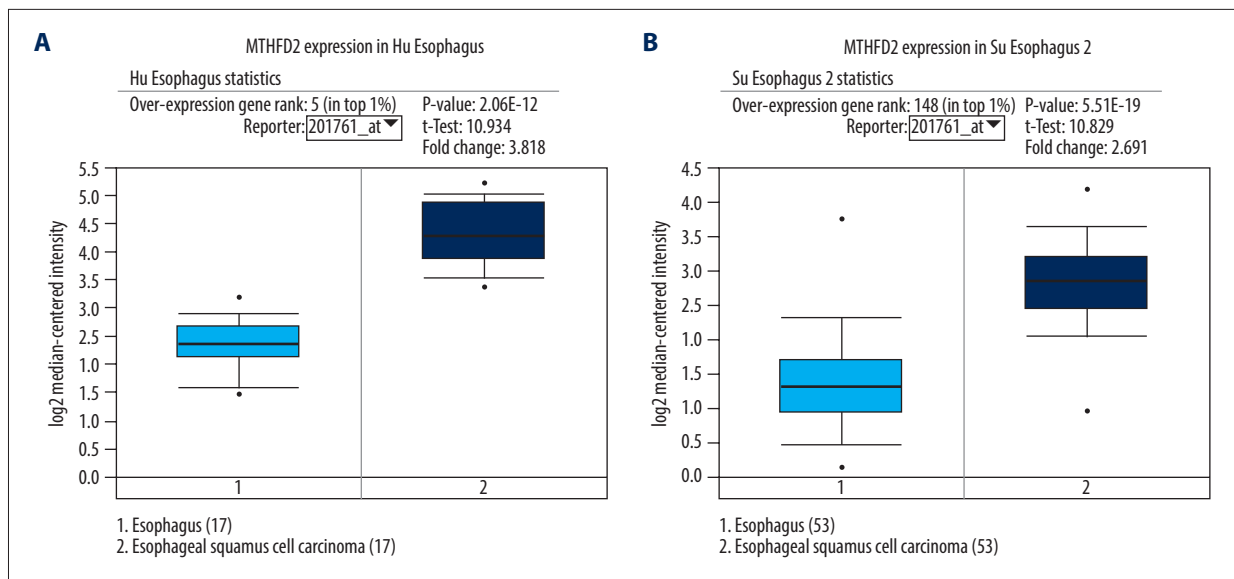
Figure 3. The Oncomine database predicts the results: (**A**) MTHFD2 is associated with esophageal squamous cell carcinoma (ESCC) (*P*<0.001). (**B**) MTHFD2 is associated with esophageal squamous cell carcinoma (ESCC) (*P*<0.001).

1 point (1–25% of the positive cells), 2 points (26–50% of the positive cells), 3 points (51–75% of the positive cells), and 4 points (76–100% of the positive cells) (Figure 2). Subsequently, by calculating the product of staining intensity and area score, patients were divided into the following 2 different expression groups: ≤6 was the low-expression group and >6 was the high-expression group [12].

The results of ki67 and p53 were determined as described previously [13,14]. Positive staining was defined as neutral or strong brown staining. The determination result of p53 was as follows: ≤15% was negative (0 points), 15–40% was medium expression (1 point), and >40% was high expression (2 points). Ki67 was determined as follows: ≤20% was negative (0 points), 21–50% was medium expression (1 point), and >50% was high expression (2 points).

Statistical analysis

We used SPSS 22.0 (IBM, Armonk, NY) software for statistical analysis. *P* value less than 0.05 was defined as statistical significance. Overall survival (OS) was calculated as the time from the start of surgery to the time of death or to the end of follow-up. The chi-squared test was used to analyze the relationship between MTHFD2 expression and clinicopathological parameters. Kaplan-Meier and log-rank tests were used to univariate survival analysis. The correlation between the 2 variables was calculated by Spearman's rank correlation analysis. Multivariate analysis of prognostic factors was performed by Cox multivariate analysis.

Results

Bioinformatics prediction results

The Oncomine database was used to predict the expression level of MTHFD2 mRNA in ESCC and normal esophageal tissue. Compared with the normal group, the expression level of MTHFD2 mRNA was significantly higher in ESCC tissues (*P*<0.05) (Figure 3A, 3B).

Expression of MTHFD2 and its relationship with clinicopathological parameters

The expression of MTHFD2 in patients with ESCC was detected by immunohistochemical staining. Immunohistochemistry of the pathological specimens of 78 ESCC patients suggested that the proteins expressed by MTHFD2 were mainly distributed in cytoplasm. In accordance with the immunohistochemical result, MTHFD2 was highly expressed in ESCC specimens at a rate of 74.36%, but not in normal esophageal tissue (*P*<0.001, Table 1, Figure 4A, 4B). The relationship between MTHFD2 expression levels and ESCC patients' clinicopathological parameters are listed in Table 2. The results showed that the expression of MTHFD2 was significantly correlated with the pathological grading (*P*=0.02) and TNM stage (*P*=0.019) of patients. However, there were no significant correlations between the MTHFD2 protein expression levels and the patient age, lymph node metastasis, or sex.

Table 1. Differences in expression of MTHFD2 between esophageal squamous cell carcinoma (ESCC) and adjacent normal tissue.

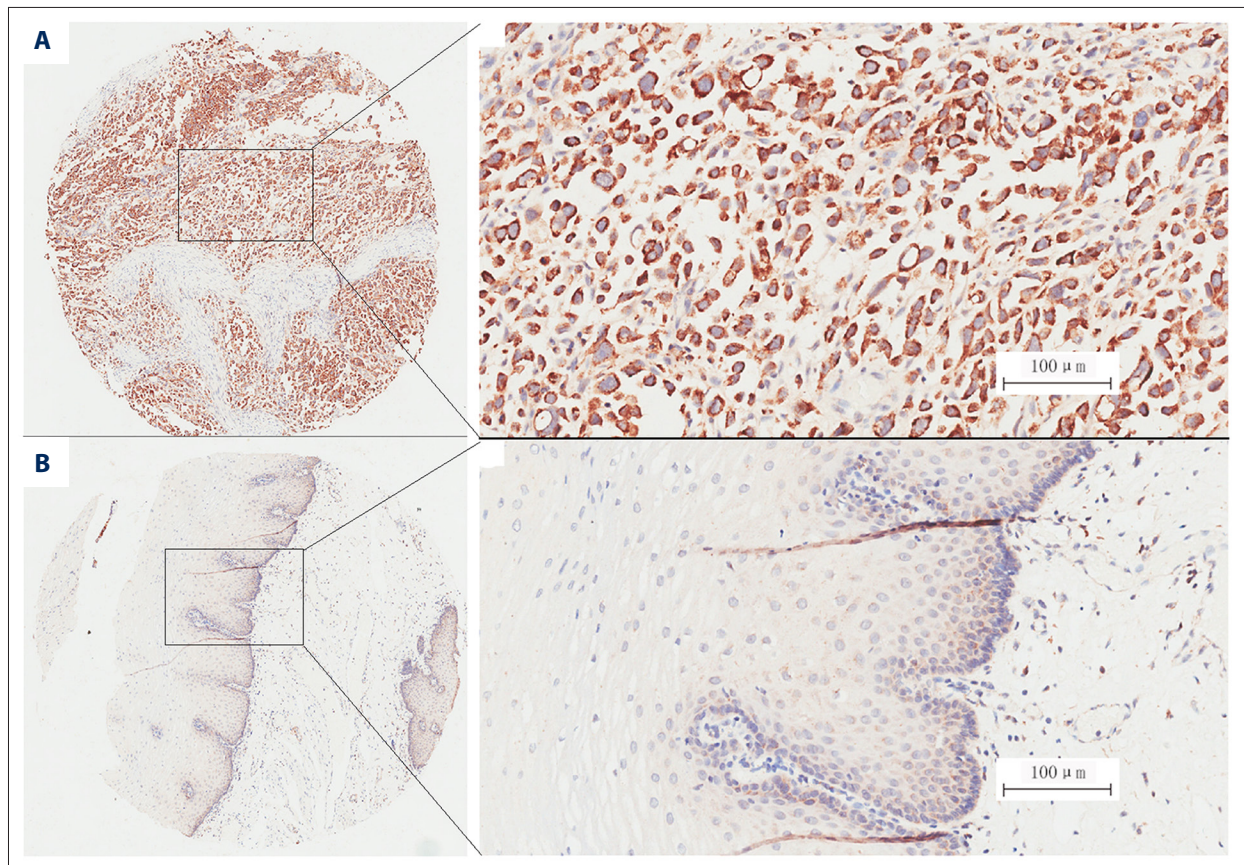| Tissues	Sum	MTHFD2 expression		High rate%	P
		Low	High		
ESCC tissues	78	20	58	74.36%	0.000
Paracarcinomatous tissues	61	61	0	0.00%	

Figure 4. Immunohistochemistry in esophageal squamous cell carcinoma (ESCC) and adjacent normal tissues: (**A**) MTHFD2 was expressed in cytoplasm and highly expressed in ESCC. (**B**) The expression of MTHFD2 was lower in the paracancerous tissues.

Expression of MTHFD2 and clinical prognosis of patients

Kaplan-Meier survival analysis and log-rank tests were used to explore the relationship between MTHFD2 expression and patient prognosis. Patients with high MTHFD2 expression had shorter OS than patients with low MTHFD2 expression (95% CI 38.227–71.473 *vs.* 22.424–38.748, *P*=0.006; Figure 5A). TNM stage was correlated with the prognosis of patients, and patients with TNM stage III/IV had worse OS than those with TNM stage I/II (95% CI 36.374–60.826 *vs.* 15.932–29.280, *P*=0.004; Figure 5B). However, sex, age, pathological grading, and presence of lymph node metastasis were not associated with OS (*P*>0.05, Figure 5C–5F). Furthermore, Cox multivariate regression analysis showed that high expression of MTHFD2 was closely associated with the prognosis of patients, which

could be used as an independent prognostic indicator for predicting ESCC (Table 3).

Relationship between high expression of MTHFD2 and ki67 and p53 in ESCC

We also used immunohistochemical staining to assess the expression of ki67 and p53 in ESCC. As shown in Figure 6, tumors with high expression of MTHFD2 had significantly higher expression of ki67 and p53. Spearman's rank correlation analysis showed that the expression of MTHFD2 was significantly positively correlated between ki67 (*r*=0.441, *P*<0.001, Table 4) and p53 (*r*=0.249, *P*=0.028, Table 5) in ESCC tissues.

Table 2. Relationship between clinicopathological features and MTHFD2 expression in 78 esophageal squamous cell carcinoma (ESCC) patients.

Clinical characteristics	Sum	MTHFD2 expression		χ^2	P
		Low	High		
Sex					
Female	19	5	14	0.000	1.000
Male	59	15	44		
Age (years)					
≤65	39	11	28	0.269	0.604
>65	39	9	30		
Pathological grading					
I/II	54	18	36	5.447	0.020
III	24	2	22		
Lymphatic metastasis					
No	40	13	27	2.026	0.155
Yes	38	7	31		
TNM stages					
I/II	45	16	29	5.484	0.019
III/IV	33	4	29		

Discussion

Diagnosis and treatment of esophageal squamous cell carcinoma currently mainly focuses on early detection, early surgery, and radical radiotherapy. The clinical research results of targeted therapy drugs for patients with advanced esophageal squamous cell carcinoma are not satisfactory. The heterogeneity of esophageal cancer and the emergence of drug resistance in the process of treatment can lead to poor efficacy. At present, the underlying molecular mechanism of ESCC pathogenesis is still unclear. Thus, to improve the survival time of ESCC patients, it is urgent to find molecular targets and biomarkers that predict prognosis in the treatment of advanced esophageal squamous cell carcinoma.

MTHFD2 is closely involved in folate metabolism during cell proliferation. Tumor cells have a rapid anabolic process that is inseparable from folate metabolism [15]. MTHFD2 has been repeatedly confirmed at the protein level to be associated with tumorigenesis, progression, and prognosis of patients, including those with highly aggressive pancreatic cancer [16]. In the present study, we observed that MTHFD2 was expressed at higher levels in ESCC tissue than in esophageal normal tissues. Therefore, we speculated that MTHFD2 plays a vital role in tumor initiation and progression. MTHFD2 was recently reported to be closely involved in the rapid growth of cells, such as embryonic cells and cancer cells, by synthesizing high levels of purine [7,8]. Mounting evidence shows that MTHFD2 is

a cancer-related metabolic enzyme. Researchers have shown that the expression of MTHFD2 mRNA and protein are upregulated in rapidly proliferating malignant tumor cells [12,17]. Moreover, there is evidence that inhibiting the expression of MTHFD2 effectively decreases the proliferation of tumor cells. For instance, Selcuklu et al. found that knockout of the MTHFD2 gene leads to the death of breast cancer cells and reduces invasion and migration [18]. Similarly, MTHFD2 was reported to promote the growth and metastasis of colorectal cancer cells, and MTHFD2 knockdown clearly inhibits tumor growth [19]. Furthermore, recent studies have demonstrated that many micro-RNAs in tumor cell pathways play an antitumor role by inhibiting the expression of MTHFD2, such as mir-940 [20], mir-504-3p [21], and mirRNA-92a [22]. The studies above indicate that MTHFD2 is closely involved in the occurrence and development of tumors. However, most previous studies did not clarify the relationship between MTHFD2 expression and tumor prognosis and/or clinicopathological parameters of ESCC.

Through bioinformatics, we observed that the expression level of MTHFD2 mRNA in ESCC tissues was significantly higher than that in the normal group. To verify this result, we conducted this study. The experimental results objectively showed that MTHFD2 was highly expressed in ESCC compared with the corresponding normal tissues, which was consistent with the bioinformatics results at the protein level. Overexpression of MTHFD2 was detectable in 58 of 78 (74.36%) tumor tissue
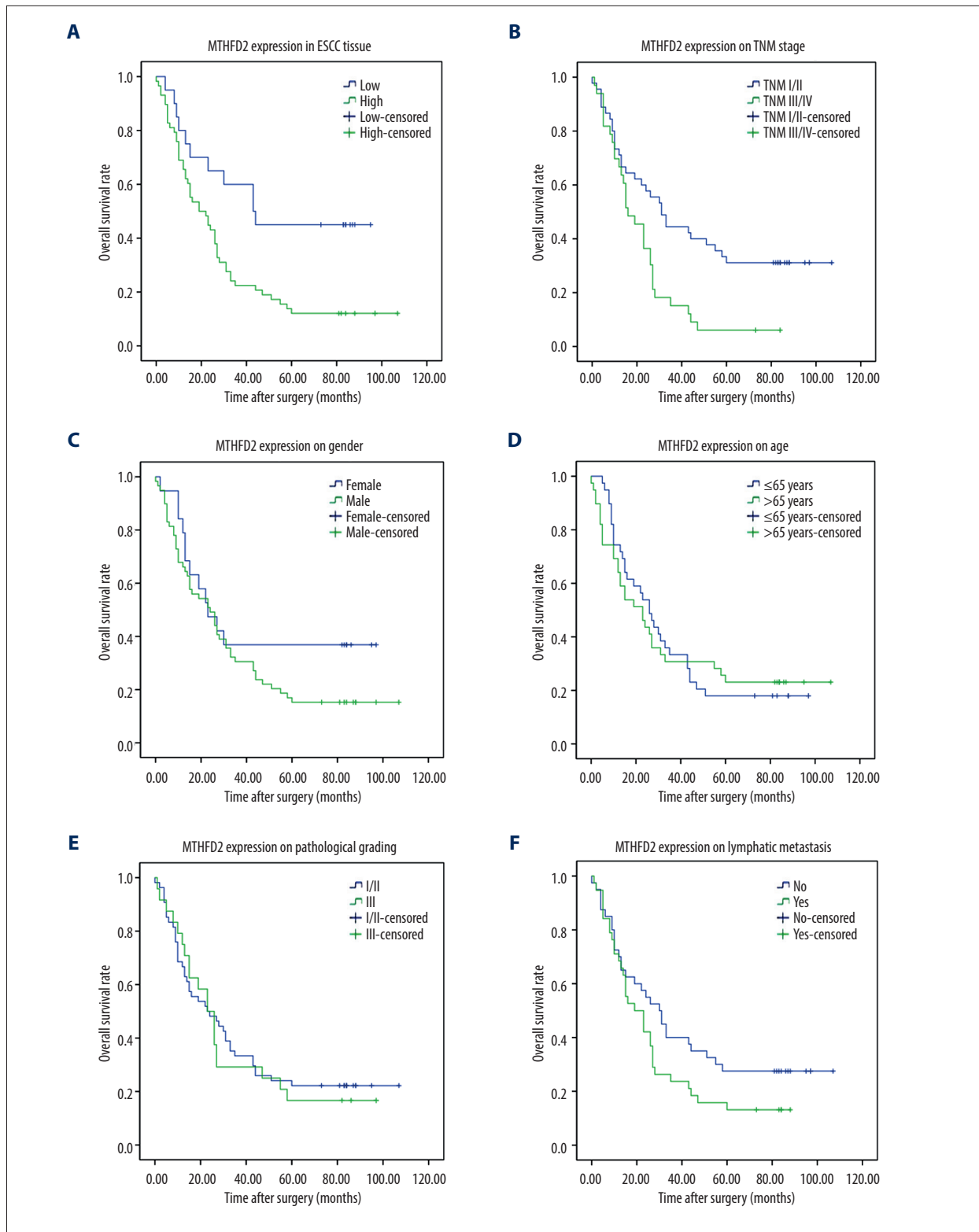
Figure 5. Kaplan-Meier method was used to estimate the overall survival (OS) of esophageal squamous cell carcinoma (ESCC) patients. (**A**) OS curve of ESCC patients based on MTHFD2 expression. (**B**) OS curve of ESCC patients based on TNM stages. (**C**) OS curve of ESCC patients based on sex. (**D**) OS curve of ESCC patients based on age. (**E**) OS curve of ESCC patients based on pathological grading. (**F**) OS curve of ESCC patients based on lymphatic metastasis.

Table 3. Univariate and multivariate analyses of factors correlated with overall survival (OS).

| Variable	Univariate analysis		P value	Multivariate analysis		P value
	Estimate	95% CI		HR	95% CI	
MTHFD2						
Low	43.000	22.547–63.453	0.006	2.205	1.135–4.283	0.020
High	19.000	9.049–28.951				
TNM stages						
I/II	31.000	21.799–40.201	0.004	1.930	1.145–3.251	0.014
III/IV	16.000	9.670–22.330				
Age (years)						
≤65	26.000	18.658–33.342	0.909			
>65	23.000	11.813–34.187				
Sex						
Female	23.000	11.625–34.375	0.161			
Male	24.000	13.248–34.752				
Pathological grading						
I/II	23.000	8.597–37.403	0.759			
III	23.000	16.279–29.721				
Lymphatic metastasis						
No	30.000	19.153–40.847	0.115			
Yes	19.000	9.334–28.666				
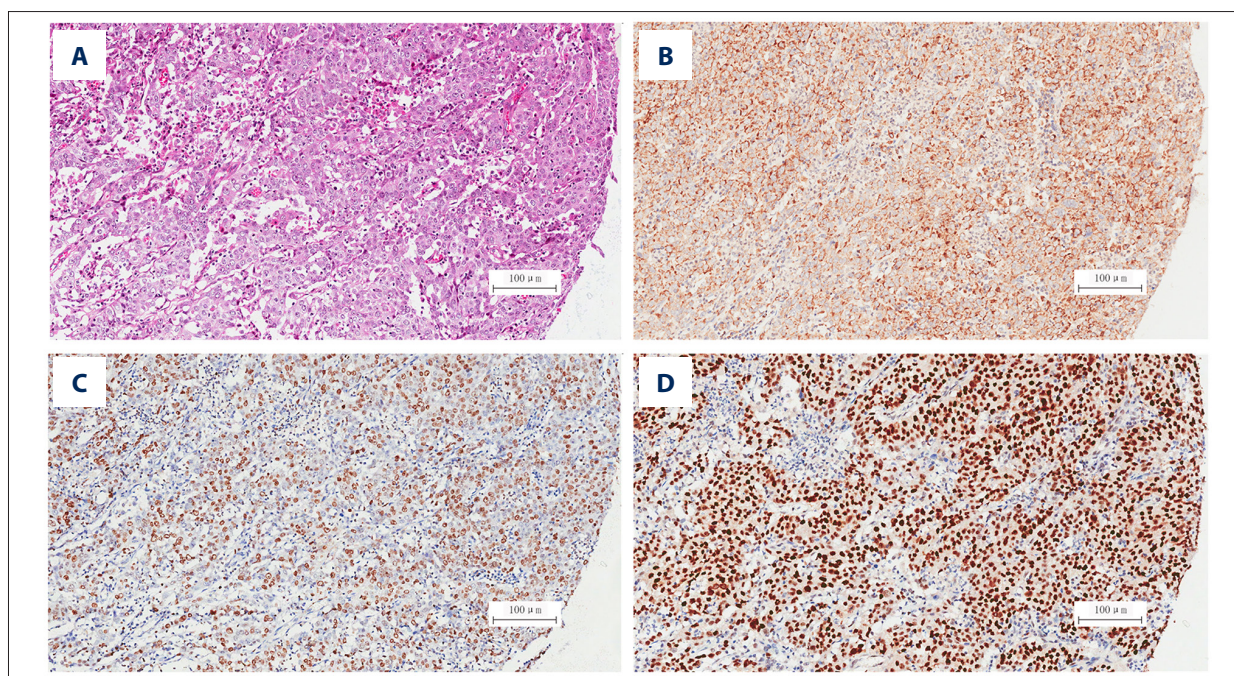
Figure 6. HE staining and immunohistochemistry were performed on the same tissues. (**A**) HE staining of tumor tissue showed abnormal cell morphology. (**B**) MTHFD2 was overexpressed in the pathological specimens of esophageal squamous cell carcinoma (ESCC). (**C**) Ki-67 was overexpressed in the pathological specimens of esophageal squamous cell carcinoma (ESCC). (**D**) P53 was overexpressed in the pathological specimens of esophageal squamous cell carcinoma (ESCC).

Table 4. The expression of MTHFD2 and ki67 in tumor tissues.

| | | MTHFD2 expression | | r | P |
		Low	High		
ki67	Low	10	5	0.441	<0.001
	High	10	53		

Table 5. The expression of MTHFD2 and p53 in tumor tissues.

| | | MTHFD2 expression | | r | P |
		Low	High		
p53	Low	7	15	0.249	0.028
	High	13	43		

samples. Furthermore, we observed that MTHFD2 overexpression was significantly correlated with the pathological grading and TNM stage of ESCC patients, suggesting that MTHFD2 is associated with the occurrence, development, and metastasis of ESCC. Further assessment demonstrated that OS was better in patients with low expression of MTHFD2 than in patients with high expression. In addition, Cox multivariate survival analysis revealed that MTHFD2 was an independent unfavorable predictor for OS of ESCC patients. In the future, the MTHFD2 may become a potential biomarker for ESCC treatment and prediction of prognosis of patients.

At present, ki67 is the most widely used markers of tumor proliferation. As a tumor suppressor gene, p53 regulates cell proliferation, but the mutant p53 is closely involved in the proliferation of various tumor cells and is related to poor prognosis of patients [23]. A previous study showed that ki67 and p53 were related to the occurrence and development of esophageal cancer [24]. Thus, to verify whether there is a relationship between MTHFD2 protein and tumor proliferation, we compared the protein expressions of MTHFD2, ki67, and p53. The results indicated that tumor tissues with high expression of MTHFD2 expressed higher ki67 and p53 than those with low MTHFD2. Further analysis by Spearman's rank correlation test showed that MTHFD2 was positively correlated with ki67.

Interestingly, MTHFD2 was also positively correlated with p53. Our results suggest that the high expression of MTHFD2 promotes the proliferation of tumor cells and inhibits apoptosis, resulting in poor prognosis of patients.

Of course, the present study has certain limitations. This study was only a retrospective study with a small sample size, which might be statistically biased. Larger experimental studies may be needed to obtain more accurate results. In addition, we only used the immunohistochemical staining, which is a semi-quantitative method, and some other quantitative methods like Western blot or qRT-PCR analysis are needed for more stronger evidence.

Conclusions

We found that MTHFD2 was highly expressed in ESCC and was closely related to poor prognosis. The study shows that MTHFD2 is an independent risk factor in ESCC, and is a potential biomarker for predicting ESCC in the future.

Conflicts of interest

None.

References:

1. Ferlay J, Shin HR, Bray F et al: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer, 2010; 127: 2893–917

2. Torre LA, Bray F, Siegel RL et al: Global cancer statistics, 2012. Cancer J Clin, 2015; 65: 87–108

3. Arnold M, Soerjomataram I, Ferlay J, Forman D: Global incidence of oesophageal cancer by histological subtype in 2012. Gut, 2015; 64: 381–87

4. Scrimgeour KG, Huennekens FM: Occurrence of a DPN-linked, N5, N10-methylene tetrahydrofolic dehydrogenase in Ehrlich ascites tumor cells. Biochem Biophys Res Commun, 1960; 2: 230–33

5. Maruyama K, Sugano S: Oligo-capping: A simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. Gene, 1994; 138: 171–74

6. Christensen KE, Mackenzie RE: Mitochondrial methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, and formyltetrahydrofolate synthetases. Vitam Horm, 2008; 79: 393–410

7. Di Pietro E, Sirois J, Tremblay ML, MacKenzie RE: Mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase is essential for embryonic development. Mol Cell Biol, 2002; 22: 4158–66

8. Di Pietro E, Wang XL, MacKenzie RE: The expression of mitochondrial methylenetetrahydrofolate dehydrogenase-cyclohydrolase supports a role in rapid cell growth. Biochim Biophys Acta, 2004; 1674: 78–84

9. Liu X, Huang Y, Jiang C et al: Methylenetetrahydrofolate dehydrogenase 2 overexpression is associated with tumor aggressiveness and poor prognosis in hepatocellular carcinoma. Dig Liver Dis, 2016; 48: 953–60

10. Liu F, Liu Y, He C et al: Increased MTHFD2 expression is associated with poor prognosis in breast cancer. Tumour Biol, 2014; 35: 8685–90

11. Lin H, Huang B, Wang H et al: MTHFD2 overexpression predicts poor prognosis in renal cell carcinoma and is associated with cell proliferation and vimentin-modulated migration and invasion. Cell Physiol Biochem, 2018; 51: 991–1000

12. Nilsson R, Jain M, Madhusudhan N et al: Metabolic enzyme expression highlights a key role for MTHFD2 and the mitochondrial folate pathway in cancer. Nat Commun, 2014; 5: 3128

13. Hage M, Siersema PD, Vissers KJ et al: Molecular evaluation of ablative therapy of Barrett's oesophagus. J Pathol, 2005; 205: 57–64

14. Sikkema M, Kerkhof M, Steyerberg EW et al: Aneuploidy and overexpression of Ki67 and p53 as markers for neoplastic progression in Barrett's esophagus: A case-control study. Am J Gastroenterol, 2009; 104: 2673–80

15. Cairns RA, Harris IS, Mak TW: Regulation of cancer cell metabolism. Nat Rev Cancer, 2011; 11: 85–95

16. Noguchi K, Konno M, Koseki J et al: The mitochondrial one-carbon metabolic pathway is associated with patient survival in pancreatic cancer. Oncol Lett, 2018; 16: 1827–34

17. Tedeschi PM, Vazquez A, Kerrigan JE, Bertino JR: Mitochondrial methylenetetrahydrofolate dehydrogenase (MTHFD2) overexpression is associated with tumor cell proliferation and is a novel target for drug development. Mol Cancer Res, 2015; 13: 1361–66

18. Selcuklu SD, Donoghue MT, Rehmet K et al: MicroRNA-9 inhibition of cell proliferation and identification of novel miR-9 targets by transcriptome profiling in breast cancer cells. J Biol Chem, 2012; 287: 29516–28

19. Ju HQ, Lu YX, Chen DL et al: Modulation of redox homeostasis by inhibition of MTHFD2 in colorectal cancer: Mechanisms and therapeutic implications. J Natl Cancer Inst, 2018 [Epub ahead of print]

20. Xu T, Zhang K, Shi J et al: MicroRNA-940 inhibits glioma progression by blocking mitochondrial folate metabolism through targeting of MTHFD2. Am J Cancer Res, 2019; 9: 250–69

21. Li SM, Zhao YQ, Hao YL, Liang YY: Upregulation of miR-504-3p is associated with favorable prognosis of acute myeloid leukemia and may serve as a tumor suppressor by targeting MTHFD2. Eur Rev Med Pharmacol Sci, 2019; 23: 1203–13

22. Gu Y, Si J, Xiao X et al: MiR-92a inhibits proliferation and induces apoptosis by regulating methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) expression in acute myeloid leukemia. Oncol Res, 2017; 25: 1069–79

23. Smith DR, Ji CY, Goh HS: Prognostic significance of p53 overexpression and mutation in colorectal adenocarcinomas. Br J Cancer, 1996; 74: 216–23

24. Binato M, Gurski RR, Fagundes RB et al: P53 and Ki-67 overexpression in gastroesophageal reflux disease – Barrett's esophagus and adenocarcinoma sequence. Dis Esophagus, 2009; 22: 588–95