Original Article

Overexpression of Cytidine Triphosphate Synthetase 1 *(CTPS1)* and Deoxythymidylate Kinase (*DTYMK)* in Patients with Newly Diagnosed Esophageal Squamous Cell Carcinoma

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What's Known

- Increased pyrimidine metabolism is characterized by dysregulation of the rate-limiting enzymes of this pathway.
- Emerging evidence highlighted the critical role of uridine cytidine kinase 2 *(UCK2),* deoxythymidylate kinase *(DTYMK),* and cytidine triphosphate synthetase 1 *(CTPS1)* in tumorigenesis and cancer progression.
- Bioinformatics analyses revealed that expression of *UCK2, CTPS1,* and *DTYMK* was increased in esophageal squamous cell carcinoma (ESCC).

What's New

- For the first time, the present study found that *CTPS1* and *DTYMK* were overexpressed in ESCC tissues compared to adjacent healthy tissue, which could provide valuable insights for the development of esophageal cancer (EC) treatment strategies.
- Although *UCK2* expression was elevated in tumor tissues compared with surrounding non-tumor tissues, this difference was not statistically significant.

Abstract

Background: Increased pyrimidine metabolism is characterized by dysregulation of the rate-limiting enzymes in this pathway, including uridine cytidine kinase 2 *(UCK2)*, deoxythymidylate kinase *(DTYMK)*, and cytidine triphosphate synthetase 1 *(CTPS1)*. Emerging evidence highlighted the critical role of these enzymes in cancer progression.

Methods: This cross-sectional study was conducted at Golestan University of Medical Sciences (Gorgan, Iran) in 2023. Initially, the Gene Expression database of Normal and Tumor tissue (GENT2) and the OncoDB database were checked to investigate the expression of these genes in esophageal cancer (EC) tissues. Subsequently, 34 pairs of newly diagnosed esophageal squamous cell carcinoma (ESCC) tissues and matched adjacent healthy tissues were collected. Real-time PCR was performed to measure the expression of these genes in the collected tissues. Statistical analyses were performed using SPSS version 22 and GraphPad Prism version 8. The Mann-Whitney test and the Wilcoxon matched-pairs signed-rank test were used to compare gene expression. $P<0.05$ was considered statistically significant.

Results: The results demonstrated that *CTPS1* (1.47 and 0.246-6.964, P=0.035) and *DTYMK* (1.62 and 0.733-5.278, P=0.009) expression levels in tumor tissue were higher than those in adjacent healthy tissues. The expression level of the *UCK2* gene (1.32 and 0.278-3.834, P=0.052) was elevated in tumor tissues compared with surrounding non-tumor tissues. However, this difference was not statistically significant. Furthermore, *DTYMK* expression was significantly higher in females (3.73 and 1.505-11.313) than in males (0.81 and 0.213-1.624, P=0.007).

Conclusion: *CTPS1* and *DTYMK* were overexpressed in ESCC tissues compared with adjacent healthy tissue, which could provide valuable insights for the development of ESCC treatment strategies.

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Keywords ● *CTP synthetase1* ● *Deoxythymidylate kinase* ● Esophageal cancer ● *Uridine-cytidine kinase 2* ● Pyrimidine synthesis

Introduction

In 2020, esophageal cancer (EC) ranked seventh in incidence and sixth in overall mortality worldwide.[1] Esophageal cancers are primarily classified into two main histological types, including esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC).[2] ESCC typically involves the middle and upper part of the esophagus and is considered the most common histological subtype of EC.[3] In 2020, the global incidence of EC exceeded 600,000 cases, and mortality reached 540,000.[4] Northeastern Iran is recognized as one of the regions with the highest incidence of EC worldwide. Specifically, the incidence of ESCC is notably high in Golestan Province, Iran.[5, 6] Despite recent advances in the diagnosis and treatment of ESCC, the 5-year survival rate remains below 20%.[7] In addition, the abnormal expression of certain genes may contribute to the pathogenesis of this disease. Consequently, identifying novel biomarkers and therapeutic targets is essential for improving clinical outcomes and advancing EC research.

One of the prominent characteristics of cancer cells is metabolic reprogramming, which contributes to tumor growth and progression. Tumor cells reprogram metabolic pathways due to increased demand for nucleotides and other essential building blocks for cell survival and rapid growth.[8, 9] Pyrimidine metabolism is frequently altered during cancer progression, a change reflected by the dysregulation of the rate-limiting enzymes (RLE) in this pathway.[10, 11] Altered expression of RLE in pyrimidine biosynthesis has been observed in various cancers. Uridine cytidine kinase 2 *(UCK2)* is an RLE in the salvage pathway of pyrimidine biosynthesis. It can phosphorylate cytidine and uridine and convert them to monophosphate forms.[12] Several studies demonstrated that *UCK2* is upregulated in cancer tissues.[13-15] Cytidine triphosphate synthetase 1 *(CTPS1)* catalyzes the rate-limiting step of CTP synthesis using UTP, Glutamine, and adenosine triphosphate (ATP).[16] Furthermore, *CTPS1* was shown to be upregulated in tumor cells.[17-19] *DTYMK* catalyzes the generation of deoxythymidine diphosphate (dTDP) from deoxythymidine monophosphate (dTMP). It represents the first convergent step of salvage and *de novo* pathways, leading to the production of dTTP, an essential nucleotide for DNA synthesis. In recent years, the roles of *DTYMK* in cancer development and its relationship with cell signaling pathways have attracted considerable research interest.[20] Reports indicated that *DTYMK* knockdown reduced tumor growth and formation in nude mice with hepatocellular carcinoma (HCC) transplanted carcinoma.[21] A previous study focused on gene expression changes using different datasets in ESCC. Based on these bioinformatics data, the expression of *UCK2, CTPS1, and DTYMK* has been reported to be increased in ESCC.[22]

However, the expression profiles of these pyrimidine metabolism-related genes in ESCC remain unclear. Therefore, we aimed to examine the expression of *UCK2, CTPS1, and DTYMK* in ESCC tissues compared with adjacent healthy tissues, with the goal of identifying potential therapeutic targets to improve clinical outcomes.

Materials and Methods

GENT2 and OncoDB Databases

The GENT2 and OncoDB databases are online platforms used to examine gene expression patterns in various cancers and healthy tissues. Initially, the expression of *UCK2, CTPS1, and DTYMK* genes was investigated in 184 EC tissues compared with 13 normal tissues, based on ribonucleic acid (RNA) sequencing data from the OncoDB database. Moreover, the expression of these genes was explored in 236 EC tissues relative to 24 healthy tissues using microarray data from the GENT2 database, which included the following datasets: GSE26886, GSE33810, GSE7307, GSE2109, GSE45670, GSE63941, GSE17351, GSE43346, GSE21293, GSE22954, GSE34111, GSE51021, and GSE42363.

Tissue Samples

The sample size of this study was calculated based on a pilot study on 10 tissue samples using G*Power 3.1.9.2 software (Heinrich Heine University Düsseldorf, Düsseldorf, Germany). A one-tailed Wilcoxon matched-pairs signed-rank test was selected, with statistical power (1-β) set to 0.8, and the α error probability set to 0.05. The mean fold change±SD obtained from the pilot study was 4.835±10.484 for *DTYMK*, 10.517±20.031 for *CTPS1*, and 4.174±9.854 for *UCK2*. Based on these parameters, a minimum sample size of 28 pairs was estimated. However, 34 subjects were ultimately included in this study.

In this cross-sectional study, 34 pairs of newly diagnosed ESCC and adjacent healthy tissue samples were obtained from Golestan Cancer Bio Bank (Gorgan, Iran) and the Iran National Tumor Bank of the Cancer Institute at Imam Khomeini Hospital (Tehran, Iran). This study was conducted at Golestan University of Medical Sciences (Gorgan, Iran) in 2023.

Patients with newly diagnosed ESCC were included. Individuals with comorbidities or those providing samples of inadequate quality that would disrupt data interpretation were excluded. All patients provided written informed consent, and the study was approved by the Ethics Committee of Golestan University of Medical Sciences (approval code: IR.GOUMS.REC.1401.395).

cDNA Synthesis

First, the tumor and adjacent healthy tissues were pulverized using liquid nitrogen. Total RNA was extracted from the tissues using the RNX-PLUS kit (SinaClon, Iran) according to the manufacturer's instructions. The concentration of the purified RNA was measured using a NanoDrop spectrophotometer (DeNovix, USA). Potential DNA contamination was removed from the RNA samples using DNase I (Thermo Fisher Scientific, USA). Subsequently, cDNA was synthesized from 1000 ng of total RNA using random hexamer and oligo (dT) primers, according to the Yekta Tajhiz Azma Kit protocol.

Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR was performed on an Applied Biosystems (ABI) instrument (Applied Biosystems, USA), and the sequences of the corresponding primers are shown in table 1. The RT-qPCR was done using SYBR Green master mix (Ampliqon, Denmark) under the following conditions: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 62 °C, and 40 s at 72 °C. The relative gene expression was normalized to 18S rRNA (as an internal reference gene) and calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

After assessing the normality of the data using the Shapiro-Wilk test, the Mann-Whitney U test was performed to compare the expression of all three genes between tumor and normal tissue groups based on the database-derived data. Subsequently, the Wilcoxon matched-pairs signed rank test was used to analyze the expression changes (fold change) of the *UCK2, DTYMK, and CTPS1* genes in tumor tissue compared with adjacent healthy tissues. Moreover, the Mann-Whitney U test was used to compare gene expression (fold change) across different subgroups based on sex, age, grade, and tumor size. Spearman's rank correlation analysis was used to evaluate the correlation between the expression of *DTYMK*, *UCK2*, and *CTPS1* genes. $P<0.05$ was considered statistically significant.

Results

Examination of Gene Expression Databases

The expression pattern of the *UCK2*, *DTYMK*, and *CTPS1* genes in EC and normal tissues was explored using the GENT2 and OncoDB databases. Data were reported as median and interquartile range (IQR; Q1–Q3). As shown in figure 1A, there was a significant elevation in the expression of the *UCK2, CTPS1, and DTYMK* genes in tumor tissues relative to healthy tissues, based on the OncoDB database.

Furthermore, according to the GENT2 database result, *DTYMK* expression was upregulated in EC tissues compared to normal tissues. However, no statistically significant difference was observed in *UCK2* and *CTPS1* expression between healthy and cancerous tissues (figure 1B).

Examination of Gene Expression in EC Tissues

The mean age of the study participants was 69.6±2.19 years, with 55.9% being female and 44.1% male. The mRNA expression of *UCK2, CTPS1, and DTYMK* was compared between tumor and adjacent healthy tissues. Data were reported as median and IQR (Q1-Q3). The results illustrated that *CTPS1* and *DTYMK* expression levels in EC tissue were higher than those in adjacent healthy tissues. The expression levels of the *UCK2* gene were increased in

Table 1: The sequences of primers

Genes	Sequences (5′ to 3′)
UCK2-F	GGGGCAGAATGAGGTGGACTAT
UCK2-R	GAGGTAAGGACACGGTAGAAGC
DTYMK-F	CCAGACCCCACAACTTCAGGA
DTYMK-R	CAACAGAGTGCCATCAGGACAG
CTPS1-F	GTCTCATCCATCTACCGAGTCC
CTPS1-R	CACAAGGGCAATAGAGCAGGTC
18S rRNA-F	GGCGGCTTTGGTGACTCTAG
18S rRNA-R	CGGTGCGGGAGTGGGTAATT

UCK2: Uridine cytidine kinase 2; DTYMK: Deoxythymidylate kinase; CTPS1: Cytidine triphosphate synthetase 1; 18S rRNA: 18S ribosomal RNA

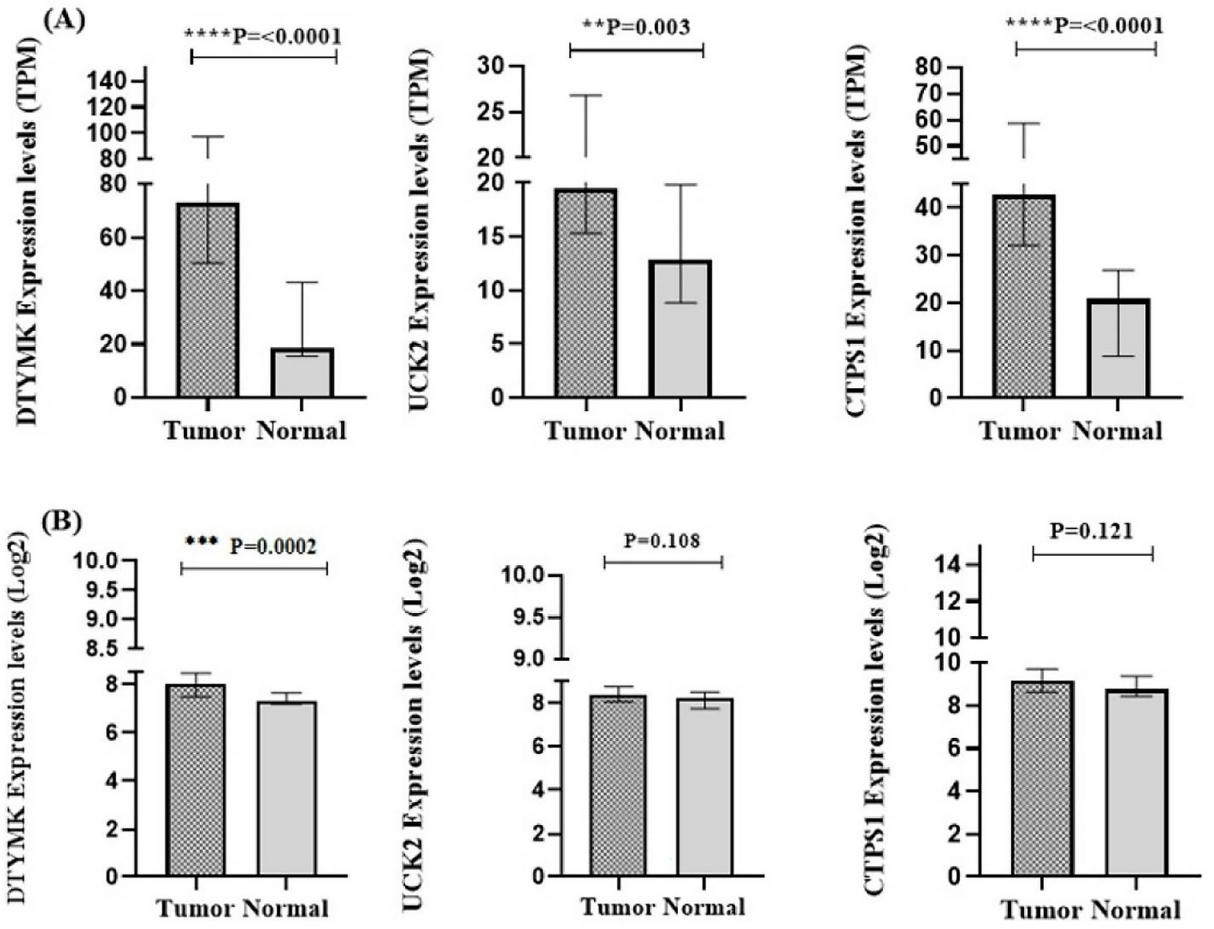

Figure 1: This figure illustrates gene expression of *DTYMK, UCK2, and CTPS1* in databases. (A) Data are reported as median and IQR (Q1-Q3). The expression of genes *UCK2* (19.47 and 15.30-26.81 vs. 12.84 and 8.82-19.83, P=0.003), *CTPS1* (42.66 and 32.08-58.63 vs. 20.84 and 8.78-26.87, P<0.0001) and *DTYMK* (73.35 and 50.48-97.52 vs. 18.80 and 15.54-43.24, P<0.0001) were upregulated in tumor tissues relative to healthy tissues in OncoDB database. (B) *DTYMK* expression was increased in tumor tissues (8.02 and 7.49-8.47 vs. 7.33 and 7.18-7.65, P=0.0002). However, no statistically significant difference was observed for *UCK2* (8.41 and 8.06-8.77 vs. 8.22 and 7.75-8.52, P=0.108) and *CTPS1* (9.18 and 8.63-9.71 vs. 8.76 and 8.43-9.37, P=0.121) expression between cancer and healthy tissues. **P=0.003, ***P=0.0002, ****P<0.0001; *UCK2: Uridine cytidine kinase 2; DTYMK: Deoxythymidylate kinase; CTPS1: Cytidine triphosphate synthetase 1;* P<0.05 was considered statistically significant.

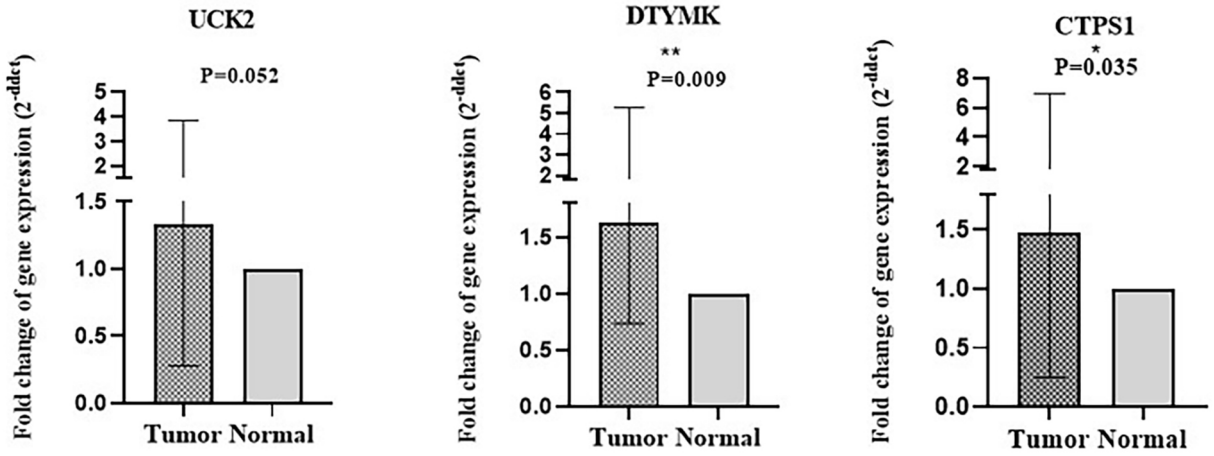

Figure 2: This figure illustrates gene expression of *DTYMK*, *UCK2*, and *CTPS1* in esophageal cancer tissues. Data are reported as median and IQR (Q1-Q3). *CTPS1* (1.47 and 0.246-6.964, P=0.035) and *DTYMK* (1.62 and 0.733-5.278, P=0.009) expression levels were enhanced in esophageal cancer tissue relative to adjacent healthy tissues. *UCK2* (1.32 and 0.278-3.834, P=0.052) was upregulated in EC tissues compared to surrounding healthy tissues. However, it was not statistically significant. *P=0.035, **P=0.009; *UCK2: Uridine cytidine kinase 2; DTYMK: Deoxythymidylate kinase; CTPS1: Cytidine triphosphate synthetase 1;* P<0.05 was considered statistically significant.

Table 2: The relationship between expression levels of *uridine cytidine kinase 2, deoxythmidylate kinase, cytidine triphosphate synthetase 1*, and clinicopathological characteristics

Variable	Genes	Group	Median	IQR (Q1-Q3)	P value
Sex	*DTYMK*	Male	0.81	0.213-1.624	0.007**
		Female	3.73	1.505-11.313	
	UCK2	Male	0.57	0.162-3.732	0.202
		Female	1.51	0.574-6.498	
	CTPS1	Male	0.80	0.117-6.093	0.274
		Female	3.02	0.563-7.366	
Age	*DTYMK*	39-64	1.62	0.759-3.681	1
		65-92	1.62	0.435-6.498	
	UCK2	39-64	1.93	0.264-4.846	0.867
		65-92	1.24	0.219-3.611	
	CTPS1	39-64	1.19	0.535-22.627	0.427
		65-92	1.62	0.101-5.656	
Grade	*DTYMK*	Well-differentiated	1.41	0.301-3.681	0.235
		Moderately/poorly-differentiated	1.86	1.368-8.905	
	UCK2	Well-differentiated	1.37	0.179-5.908	0.664
		Moderately/poorly-differentiated	1.65	0.374-3.611	
	CTPS1	Well-differentiated	0.65	0.339-4.141	0.382
		Moderately/poorly-differentiated	3.02	0.311-7.844	
Tumor size	*DTYMK*	<5 cm	2.67	0.985-13.494	0.395
		≥5 cm	1.62	0.435-5.278	
	UCK2	<5 cm	1.89	0.707-8.574	0.317
		≥5 cm	1.93	0.173-4.423	
	CTPS1	<5 cm	1.31	0.865-3.028	0.605
		≥5 cm	0.40	0.052-7.769	

UCK2: Uridine cytidine kinase 2; DTYMK: Deoxythymidylate kinase; CTPS1: Cytidine triphosphate synthetase 1; The Mann-Whitney test was used to compare gene expression (fold change) across different groupings based on sex, age, grade, and tumor size. P<0.05 was considered statistically significant.

Table 3: Spearman's correlation between *uridine cytidine kinase 2, deoxythymidylate kinase,* and *cytidine triphosphate synthetase 1*

Genes		*DTYMK*	*CTPS1*	*UCK2*
DTYMK	Correlation Coefficient	1	0.475*	0.496**
	P value	-	0.016	0.007
CTPS1	Correlation Coefficient	0.475*	1	0.333
	P value	0.016	-	0.097
UCK2	Correlation Coefficient	0.496**	0.333	1
	P value	0.007	0.097	-

UCK2: Uridine cytidine kinase 2; DTYMK: Deoxythymidylate kinase; CTPS1: Cytidine triphosphate synthetase 1; Spearman's correlation analysis was used to measure the correlation between the expression of DTYMK, UCK2, and *CTPS1* genes. P<0.05 was considered statistically significant.

cancer tissues compared to surrounding non-tumor tissues. However, this increase was not statistically significant (figure 2).

Examination of Gene Expression Based on Demographic and Pathologic Characteristics

To examine whether there was a relationship between expression levels of three target genes and pathological and demographic characteristics, including age, grade, tumor size, and sex, the studied subjects were grouped according to age (39-64 years and 65-92 years), grade (well-differentiated and moderately/poorly differentiated), tumor size (<5 cm and ≥5 cm) and sex (male and female). Our findings showed that *DTYMK* expression in females was higher than in males. No significant differences were observed in the expression levels of the three genes among other subgroups. The results are presented in table 2.

As expected, Spearman's correlation analysis revealed significant correlations between expression levels of *DTYMK, UCK2, and CTPS1*. There were significant correlations between *DTYMK and UCK2*, and between *DTYMK and CTPS1*. No correlation was observed between *CTPS1* and *UCK2* (table 3).

Discussion

This study examined the expression profile of RLE within the pyrimidine synthesis pathway in ESCC tissues and adjacent non-tumor tissues.

The findings of the present study demonstrated the upregulation of these genes in cancer tissues, suggesting a vital role for pyrimidine metabolism in the progression of EC. Metabolic reprogramming is considered one of the main characteristics of cancer. The metabolic demands for DNA replication and nucleotide synthesis are increased in cancer cells to maintain their survival, uncontrolled proliferation, and growth.[23, 24] Using bioinformatics data, Wang and others showed that the expression of genes related to pyrimidine metabolism was elevated in 80% of cancers compared to healthy tissues, which indicated a correlation between pyrimidine metabolism and cancer development.[9] Increased pyrimidine metabolism is reflected by the dysregulation of RLEs in this pathway.[10] *UCK2*, *DTYMK*, and *CTPS1* are RLEs involved in the *de novo* and salvage pathways of pyrimidine synthesis. Therefore, alterations in the expression of these enzymes could be important in cancer progression.

Initially, we explored the OncoDB and GENT2 databases to examine the expression pattern of *UCK2*, *DTYMK*, and *CTPS1* in EC. According to the OncoDB database, the expression of *UCK2, CTPS1,* and *DTYMK* was upregulated in tumor tissues compared to normal tissues. Based on the results obtained from the GENT2 database, *DTYMK* expression was increased, while no significant changes were observed in the expression of *UCK2* and *CTPS1* in tumor tissues compared to normal ones. These discrepancies might be attributed to differences in data sources, technical platforms, and sample sizes.

In the present study, the expression of *UCK2*, *DTYMK*, and *CTPS1* was analyzed in ESCC tissues and adjacent healthy tissues. Our findings demonstrated that *CTPS1* and *DTYMK* expression were upregulated in EC tissues compared with adjacent healthy tissues. In line with our findings, Zhang and others found that *DTYMK* expression was elevated in HCC tissues compared with healthy liver tissues.[25] Furthermore, *DTYMK* expression was reported to be increased 1.6-fold in HCC compared with normal tissues,[26] and upregulated in most HCC tissues as well as in HepG2, Hun7, and Hep3B HCC cell lines relative to healthy hepatic cell lines.[21] It was observed that increased *DTYMK* was associated with worse prognosis and poor survival in patients with colorectal cancer, with *DTYMK* levels correlating with cancer progression and invasion.[27] Another study showed that *DTYMK* was overexpressed in several cancers, and its upregulation was associated with more aggressive tumors.[28] Consistent with our findings, the expression of *CTPS1* was shown to be increased in triple-negative breast cancer (TNBC) tissues and cells.[17] *CTPS1* and ataxia-telangiectasia and Rad3 related (ATR) inhibition enhanced cell death and reduced tumor growth in MYC-overexpressing cancer cells.[29] The researchers showed that the expression of *CTPS1* was upregulated in breast cancer tissues and that *CTPS1* inhibition reduced migration, proliferation, and invasion in breast cancer cells.[30] Furthermore, *CTPS1* inhibition suppressed proliferation, induced apoptosis in lymphoid cells, and blocked the growth of B-cell and T-cell lymphomas *in vivo*.[31] In the present investigation, *UCK2* expression was enhanced in tumor tissues relative to surrounding non-tumor tissues. However, this difference was not statistically significant. Nevertheless, several studies reported that *UCK2* was increased in various cancers.[13, 32, 33] These discrepancies might be attributed to factors such as sample size, cancer type, technical platforms, and ethnic groups.

Few studies have reported correlations between the expressions of these target genes and demographic and pathological parameters. In this study, no significant differences were observed in the expression levels of *UCK2* and *CTPS1* concerning the clinicopathological characteristics, such as age, sex, tumor grade, and tumor size. Our results indicated that *DTYMK* expression in females was higher than in males. Consistent with some previous reports, no significant difference in *UCK2* levels was observed based on age, sex, and tumor grade in pancreatic cancer.[34] In another study, *DTYMK* levels were not associated with sex, age, tumor size, and tumor-node-metastasis (TNM) stage in hepatocellular carcinoma.[26] Conversely, Zhou and others demonstrated that increased *DTYMK* expression was significantly correlated with sex in lung adenocarcinoma.[35] Similarly, no significant correlation was found between *UCK2* expression and age, sex, tumor size, and Edmondson-Steiner grade in patients with HCC.[36] However, elevated *CTPS1* expression was correlated with larger tumor size and higher grade in breast cancer.[17] However, increased UCK2 protein levels in patients with HCC were correlated with poor differentiation grade, advanced TNM stage, and tumor size. However, no significant association was observed with age or sex.[33]

Overall, pyrimidine metabolic reprogramming facilitates cancer progression and growth, and understanding the changes in gene expression involved in this pathway could help in identifying suitable therapeutic targets. Nevertheless, the present study had several limitations. First, as

a small cross-sectional study, it failed to fully capture the molecular diversity of EC. Second, the association of these enzymes with other pathological features was not examined. Third, enzymatic activity of these rate-limiting enzymes was not measured. Moreover, expression data alone cannot confirm mechanistic roles or therapeutic applications. Thus, further mechanistic studies are required to elucidate how these enzymes interact with signaling pathways and to evaluate the effects of their inhibition on cancer treatment. Therefore, an in-depth investigation of the genes involved in pyrimidine synthesis could provide therapeutic options for EC.

Conclusion

The experimental results demonstrated that the expression of *CTPS1* and *DTYMK* was significantly increased in ESCC tissues compared with adjacent healthy tissues. These findings could provide valuable insights into the upregulation of genes involved in pyrimidine metabolism and highlight their importance as therapeutic targets in EC.

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Authors' Contribution

N.H: Conceptualization, study design, experimental performance, data analysis, and drafting; M.SJ: Experimental performance, data analysis, and reviewing the manuscript; J.A: Conceptualization, study design, and reviewing the manuscript; M.ShA: Conceptualization, study design, and reviewing the manuscript; SM.J: Conceptualization, study design, experimental performance, data analysis, and reviewing the manuscript; All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of AI

The authors used ChatGPT version 5.3 (OpenAI, San Francisco, USA) to improve clarity and paraphrase some sentences. All conceptual content, data analysis, and interpretation were conducted solely by the authors.

Conflict of Interest: None declared.

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