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Original Article

Elevated expression of DTYMK is associated with poor prognosis in patients with Non-small cell lung cancer

Wei Wang[1,2,3*], Zhi-Hua Guo[1,2,3*], Xin-Peng Lu[2,3], Dong-Jiang Liao[2,3], Gui-Lin Peng[1,2,3], Xin Xu[1,2,3], Wei-Qiang Yin[1,2,3], Jian-Xing He[1,2,3]

[1]Department of Thoracic Surgery, First Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, China; [2]Guangzhou Institute of Respiratory Diseases & China State Key Laboratory of Respiratory Disease, Guangzhou 510120, China; [3]National Respiratory Disease Clinical Research Center, Guangzhou 510000, China. [*]Equal contributors.

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Abstract: Deoxythymidylate Kinase (DTYMK), a key enzyme for pyrimidine synthesis that catalyzes the phosphorylation of dTMP to produce dTDP in the presence of ATP and magnesium, has recently been considered to be associated with the progress of various human cancers. However, the clinical significance of DTYMK in Non-small cell lung cancer (NSCLC) remains unclear. In the present study, we performed immunohistochemistry analysis on human tissue microarray (TMA) to detect the DTYMK protein expression pattern, which was further validated by high-throughput sequencing data TCGA database at mRNA level. Immunohistochemistry analysis found that DTYMK protein expression in NSCLC tissues was significantly higher than those in normal lung tissues (*P*=0.000). Additionally, high DTYMK was dramatically associated with the advanced tumor status (*P*=0.000), enhanced nodal status (*P*=0.000) and high TNM stage (*P*=0.000). The mRNA expression in TCGA database showed that DTYMK was upregulated in NSCLC tissues with old age (*P*=0.018), smokers (*P*=0.017), and high TNM stage (*P*=0.005). Furthermore, Kaplan-Meier survival curves revealed that NSCLC patients with high DTYMK levels had shorter survival compared with those showing low DTYMK expression (*P*=0.012). The upregulation of DTYMK was an independent prognostic factor for NSCLC patients (HR: 0.472, 95% CI 0.251-0.887; *P*=0.020). In conclusion, our findings disclose that DTYMK may play an important role in tumor progression of NSCLC and high DTYMK may efficiently predict poor prognosis in NSCLC patients.

Keywords: Non-small cell lung cancer, deoxythymidylate kinase, clinicopathological feature, tumor promoter, prognosis

Introduction

Lung cancer is one of the most common cancers and rank as the leading cause of cancer-related deaths globally, with an estimated 228,190 new cases and 159,480 deaths in the United States in 2015 [1]. Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are main subtypes of lung cancer, and NSCLC accounting for more than 80% of all cases [2]. As a heterogeneous cancer with a complex pathogenesis disease, its natural histology is highly variable and difficult to predict. Only 20-30% of NSCLC patients are radically resectable and the majority patients succumb to the disease. Although developments in early diagnosis and novel systemic treatment have been made, NSCLC is generally diagnosed at an advanced stage and most patients showed a poor 5-year survival rate [3]. The failure of therapy and high mortality of the disease are largely attributed to late diagnosis, when metastases and recurrence are present [4]. Recent researches have discovered several molecular alterations used to predict the prognosis of NSCLC [5-8]. However, the precise mechanism underlying this malignancy has not been elucidated. Therefore, it is essential to identify novel and effective biomarkers, which can further clarify the biological characteristics of NSCLC, improve adjuvant treatments and predict prognosis.

Deoxythymidylate Kinase (DTYMK, thymidine monophosphate kinase, EC 2.7.4.9) is a key enzyme for pyrimidine synthesis that catalyzes

Table 1. Baseline information and Clinical features of all patients

Clinical Features	Experiment Type	
	TMA (%)	TCGA database (%)
Lung Cancer (Cases)	110 (100.0)	229 (100.0)
Gender		
Male	74 (67.3)	98 (42.8)
Female	36 (32.7)	124 (57.2)
Mean age	54.96±11.67	66.10±9.69
<60	61 (55.5)	52 (22.7)
≥60	49 (44.5)	154 (77.3)
Smoking status		
Nonsmoker	-	32 (14.0)
Smoker	-	180 (86.0)
Tumor status		
T1 T2	80 (72.7)	190 (83.0)
T3 T4	30 (27.3)	31 (17.0)
Nodal status		
N0	58 (52.7)	-
N1 N2 N3	52 (47.3)	-
TNM stage		
I	50 (45.5)	115 (50.2)
II/III/IV	60 (54.5)	102 (49.8)
Metastasis	1 (0.9)	8 (3.5)
Normal lung Tissue (Cases)	10	0

Note: the "-" means there are lack of relative information of patients in that cohort. Abbreviation: TMA, tissue microarray; TCGA database: the cancer genome atlas database.

the phosphorylation of dTMP to produce TDP in the presence of ATP and magnesium [9-11]; dTDP is further phosphorylated by nucleoside-diphosphate kinase (NDK) to form thymidine 5'-triphosphate (dTTP). As the substrate of DTYMK, dTMP comes either from thymidine kinase (TK) involved salvage pathway or from thymidylate synthase (TS) mediated de novo pathway [12]. Therefore, DTYMK is the first enzymatic step following the convergence of the de novo and salvage pathways in dTTP biosynthesis [9]. dTTP is an important building block in DNA synthesis, and its level is tightly controlled at different phases of the cell cycle during cell proliferation [9, 12]. Being a key enzyme in the synthesis of dTTP, DTYMK is overexpressed in various human cancers such as malignant rhabdoid tumor of the kidney [13], malignant mesothelioma [14], acute lymphoblastic leukemia [15], nasopharyngeal carcinoma [16] and pancreatic cancer [17]. However, the role of DTYMK in NSCLC has not been investigated.

In the present study, we investigate the expression of DTYMK in NSCLC and its correlations with clinicopathological characteristics and prognosis of NSCLC patients.

Materials and methods

Patients and tissue samples

For immunohistochemistry analysis, tissue microarray (TMA, n=120) including 110 lung cancer tissues and 10 normal lung tissues were obtained from Xi'an Alenabio Co, LTD (Cat No: BC041115b), including the detailed clinical information. Patients with known chemotherapy or radiotherapy before the surgery were excluded from the study. In order to investigate the expression of DTYMK at mRNA level and perform the survival analysis, the clinical information of the TCGA database including 229 lung cancer tissues was also collected. The detailed information on the clinical features of all patients in this study is classified in **Table 1**.

All 229 patients in the TCGA database were given a follow-up exam ranging from 0 to 224 months, and the median survival was 13 months. For the analysis of survival and follow-up, the date of surgery was used as the beginning of the follow-up period. The analysis endpoint for the cohort of patients was time to death or the date of last visit (if death did not occur). All patients that died from diseases other than lung cancer or unexpected events were excluded from the cohort.

Immunohistochemistry analysis

The specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 4 μm and then deparaffinized with xylene and rehydrated for further peroxidase (DAB) immunohistochemistry staining employing DAKO EnVision System (Dako Diagnostics, Switzerland). Following a brief proteolytic digestion and a peroxidase blocking of tissue slides, the slides were incubated overnight with the primary antibody against DTYMK (rabbit monoclonal antibody, ab154867, Abcam Co. Ltd.,

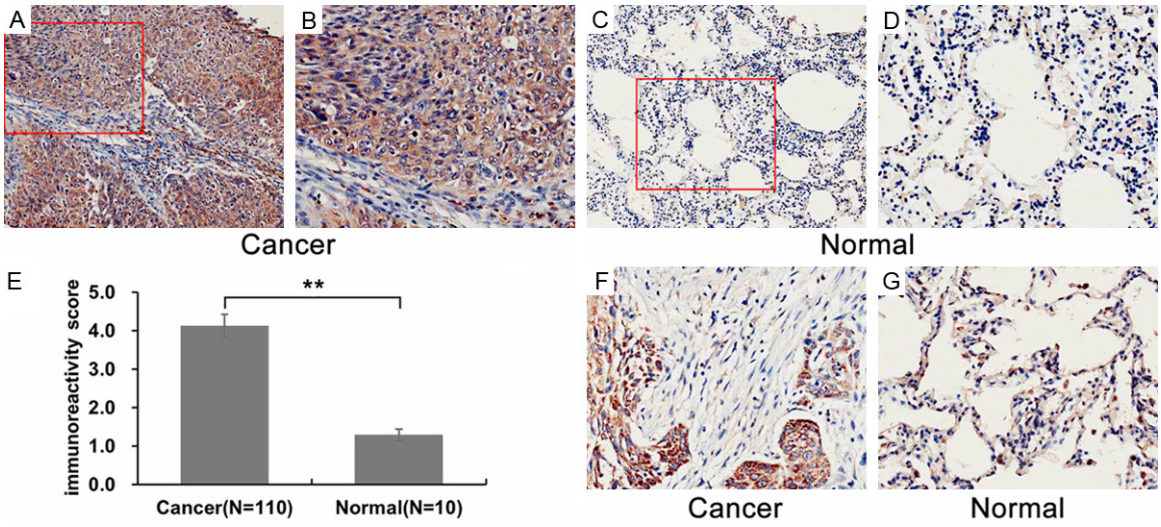

Figure 1. Immunohistochemical staining for DTYMK in NSCLC and normal lung tissues in our TMA samples. A-D. The immunohistochemistry staining indicated that DTYMK immunostainings occurred strongly in the cytoplasm in cancer cells of NSCLC tissues, but weakly or moderately in normal lung tissues. E. The expression level of DTYMK was significantly higher in NSCLC tissues than in normal lung tissues (IRS: NSCLC=4.13±3.17 vs. Normal =1.30±0.48, *P*=0.006). **mean *P*<0.01. F, G. Immunohistochemical staining of DTYMK occurred in stroma between cancer cells, but moderately in the normal lung tissue.

Table 2. Association of DTYMK expression with the clinicopathological characteristics of lung cancer in two cohorts

Clinical feature	IRS of DTYMK in our cohort				DTYMK expression in TCGA database		
	Case	Low (%)	High (%)	*P*	Case	$\bar{X}$±s	*P*
DTYMK expression							
Benign	10	10 (100.0)	0 (0.0)	0.000**	0	-	-
Cancer	110	46 (41.8)	64 (58.2)		229	641.53±456.40	
Mean age							
<60	61	29 (47.5)	32 (52.5)	0.243	52	778.79±80.34	0.018**
≥60	49	17 (34.7)	32 (65.3)		154	601.83±33.78	
Gender							
Male	74	31 (41.9)	43 (58.1)	1.000	98	692.03±46.31	0.182
Female	36	15 (41.7)	21 (58.3)		124	608.95±41.32	
Smoking status							
Nonsmoker	-	-	-	-	32	521.73±44.41	0.017*
Smoker	-	-	-		180	660.96±35.47	
Tumor status							
T1 T2	80	43 (53.8)	37 (46.3)	0.000**	190	641.99±32.54	0.664
T3 T4	30	3 (10.0)	27 (90.0)		31	680.89±96.06	
Nodal status							
N0	58	36 (62.1)	22 (37.9)	0.000**	-	-	-
N1 N2 N3	52	10 (19.2)	42 (80.8)		-	-	
TNM stage							
I	50	35 (70.0)	15 (30.0)	0.000**	115	569.31±27.64	0.005**
II/III/IV	60	11 (18.3)	49 (81.7)		102	744.24±58.06	

Note: the "-" means there are lack of relative information of patients in our cohort, *means *P*<0.05, **means *P*<0.01. Abbreviation: IRS, immunoreactivity scores; DTYMK, Deoxythymidylate Kinase; TCGA database: the cancer genome atlas database.

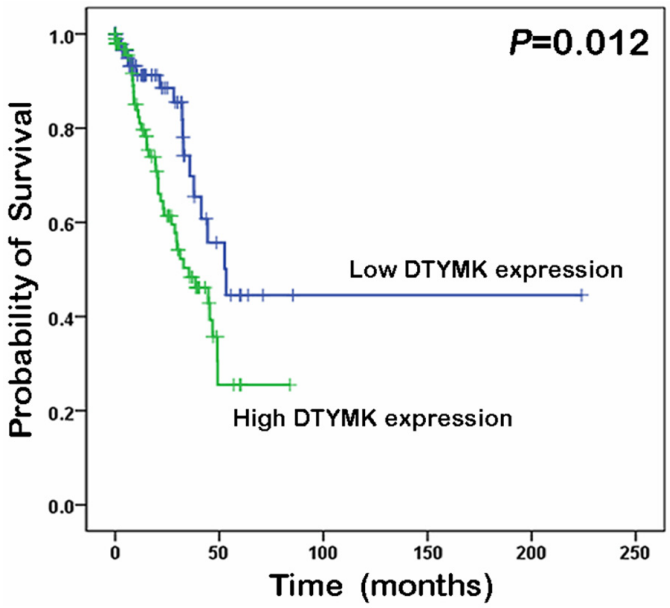

Figure 2. Kaplan-Meier survival curves of overall survival for DTYMK expression in NSCLC.

UK) at a dilution of 1:200, at 4°C. After washing, peroxidase labeled polymer and substrate-chromogen were employed in order to visualize the staining of the interested protein. In each immunohistochemistry run, negative controls were carried out by omitting the primary antibody.

Evaluation of immunostaining results

The intensity of immunostaining was scored separately by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through reevaluated by a re-examination of the staining by both pathologists to achieve a consensus score. The immunolabeling of cancer cells was evaluated. The number of positive-staining cells in five representative fields at a 400-fold was counted and the percentage of positive cells was also calculated. According to the antibody specification sheet, cytoplasmic staining was regarded as positive signals. The semi-quantitative scoring of the expression intensity in each sample was performed according to a previous report and was based on the staining intensity and percentage. The staining intensity was visually scored and stratified according to the following criteria: no staining (0 points), mild staining (1 point), moderate staining (2 points), and strong staining (3 points). The percentage scoring of immunoreactive tumor cells was defined as follows: <5% (0 points), 6-25% (1 point), 26-50% (2 points), 51-75% (3 points), and >75% (4 points). The final immunoreactivity scores (IRS) of each case were the multiplication of calculated by multiplying the two scores for the immunostaining intensity and immunostaining percentage.

Statistical analysis

SPSS 21.0 software (SPSS Inc, IL, USA) was used for statistical analysis. Pearson's Chi-squared tests and Fisher's exact test were used to analyze the association of DTYMK expression with clinicopathological characteristics. Overall survivals were analyzed using Kaplan-Meier method, and differences were assessed using log-rank test. Univariate analysis comparisons (gender, age, smoking status, tumor status, pTNM stage and DTYMK mRNA expression) and multivariate survival comparisons were performed using Cox proportional hazard regression models. The relative risks of dying were expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). Differences were statistically significant when $P<0.05$.

Results

DTYMK protein is up-regulated in human NSCLC tissues

Firstly, we detected DTYMK protein expression in TMA (**Table 1**) by IHC. As shown in **Figure 1A-D**, DTYMK immunostainings occurred strongly in the cytoplasm in cancer cells of NSCLC tissues, but weakly in benign lung tissues. Of the 110 NSCLC samples, 46 (41.8%) demonstrated low DTYMK expression, while 64 (58.2%) were highly stained for DTYMK. Furthermore, the expression level of DTYMK in NSCLC tissues was significantly higher than that in normal lung tissues (IRS: NSCLC= 4.13±3.17 vs. Normal =1.30±0.48, $P=0.006$) (**Figure 1E**).

Immunostaining results were analyzed using only the limited clinical information of the TMA.

Table 3. Prognostic value of DTYMK expression for the overall survival by Cox proportional hazards model

Variable	Beta value	HR (95% CI)	*P*
Univariate analysis			
Age (<60 y vs. ≥60 y)	-0.162	0.850 (0.482-1.501)	0.576
Gender (male vs. female)	-0.237	1.267 (0.752-2.134)	0.374
Smoking status (nonsmoker vs. smoker)	-0.296	0.744 (0.349-1.586)	0.443
Tumor status (T1-T2 vs. T3-T4)	-0.898	0.407 (0.219-0.758)	0.005**
TNM stage (I vs. II/III/IV)	-0.980	0.375 (0.217-0.651)	0.000**
DTYMK (low (N=101) vs. high (N=101))	-0.705	0.494 (0.280-0.873)	0.015*
Multivariate analysis			
Age (<60 y vs. ≥60 y)	-0.475	0.622 (0.315-1.226)	0.170
Gender (male vs. female)	0.333	1.395 (0.735-2.650)	0.309
Smoking status (nonsmoker vs. smoker)	-0.239	0.787 (0.326-1.900)	0.594
Tumor status (T1-T2 vs. T3-T4)	-0.817	0.442 (0.213-0.917)	0.028*
TNM stage (I vs. II/III/IV)	-0.739	0.478 (0.251-0.910)	0.025*
DTYMK (low (N=101) vs. high (N=101))	-0.750	0.472 (0.251-0.887)	0.020*

Note: *means $P<0.05$, **means $P<0.01$.

The results showed that the overexpression of DTYMK protein was significantly associated with advanced tumor status (P=0.000), enhanced nodal status (P=0.000) and high TNM stage (P=0.000). However, high DTYMK levels were not associated with age and gender (both $P>0.05$) (**Table 2**).

Increased expression of DTYMK is associated with the aggressive progression and poor prognosis of NSCLC in TCGA database

To validate the results of our cohort, a publicly available database (TCGA database) consisting of 229 NSCLC tissues with high-throughput sequencing data for protein coding genes (mRNA) expression data [18] was used. As shown in **Table 2**, DTYMK was upregulated in NSCLC tissues with old age (P=0.018), smoker (P=0.017), and high TNM stage (P=0.005) patients. However, high DTYMK expression was not associated with gender and tumor status (both $P>0.05$).

DTYMK serves as an independent prognostic factor for the survival of NSCLC patients

The association of DTYMK expression with the overall survival time of NSCLC patients was analyzed by Kaplan-Meier plots using the TCGA database. The median of DTYMK expression level in all NSCLC tissues of the TCGA database was used as a cutoff to divide the NSCLC tissues of each group into high and low DTYMK expression groups. As shown in **Figure 2**, the overall survival of NSCLC patients with high DTYMK expression were significantly shorter than those with low DTYMK expression (P= 0.012). In addition, the univariate analysis revealed that DTYMK expression (HR 0.494, 95% CI 0.280-0.873; P= 0.015) were significant prognostic factors for overall survival in patients with NSCLC (**Table 3**). Multivariate analysis using Cox proportional hazards model revealed that high D TYMK expression was a significant independent prognostic factor in NSCLC (HR 0.472, 95% CI 0.251-0.887; P= 0.020) (**Table 3**).

Discussion

As a heterogeneous cancer with a complex pathogenesis disease, early detection, early diagnosis and early treatment have been recognized as the keys to treating lung cancer. Therefore, it is of critical significance to identify and characterized novel markers in order to investigate new therapeutic approaches and improve patients' clinical outcome. The current study indicated that the expression of DTYMK protein was upregulated in NSCLC tissues compared to those in non-cancerous lung tissues. In addition, the overexpression of DTYMK was dramatically associated with the aggressive tumor progression of NSCLC patients, including advanced tumor status, enhanced nodal status, high TNM stage and short overall survival time. More importantly, PRC1 was identified as a potential biomarker in early diagnosis in NSCLC patients. To the best of our knowledge, clinical significance of DTYMK in the context of human NSCLC has not been reported.

DTYMK, as a dTMP kinase, could affect the DNA synthesis and is closely linked to cell pro-

liferation. It catalyzes phosphorylation of dTMP to form dTDP and dTTP, in the presence ATP and magnesium. Recent research has demonstrated that DTYMK is up-regulated in various cancers [13-17]. As for human NPCs, DTYMK was significantly overexpressed in tumor tissue compared with non-tumor tissue. Knockdown of DTYMK using lentiviral-based shRNA in TP53+/+ and TP53-/- HCT116 colon cancer cells significantly decreased the dTTP pool, augmented the DNA damage response and enhanced apoptotic induction after exposure to low-dose doxorubicin, leading to cell death [19]. So far, there have been no reports which state the relationship between the DTYMK expression and patients' clinicopathological features. But some scholars make adequate research on TK, which is the upstream gene of DTYMK on *salvage* pathway. Similar to DTYMK, TK is also an important enzyme known to be associated with DNA synthesis of cells, and overexpression in cancer [20]. Kemik et al. [21] reported that higher serum TK1 activity levels are correlated with a more advanced cancer stage and grade. Moreover, Bolayirli [22] and Liu et al. [23] showed that serum TK1 activity in patients with breast, colorectal and gastric cancer was significantly higher than that of the healthy controls, and after the completion of chemotherapy the values were lower than baseline. DTYMK and TK both belong to the enzyme in cell proliferation. And TK1 have been validated as clinically applicable prognostic markers for lymphoma and leukemias [24], non-small cell lung cancer [25], gastric cancer [23] and breast cancer [26].

In summary, our data have provided evidence that DTYMK is upregulated in human NSCLC. We also document for the first time that high DTYMK associates with the aggressive progression and poor prognosis in man with NSCLC highlighting its potential as a prognostic marker and a novel molecular target for NSCLC treatment. However, further study is necessary to gain a full understanding of the underlying molecular mechanism.

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Disclosure of conflict of interest

None.

Address correspondence to: Wei Wang and Jianxing He, Department of Cardiothoracic Surgery, The First Affiliated Hospital of Guangzhou Medical University, No. 151, Yanjiang Rd, Guangzhou 510120, China. Tel: +86 20 83062810; Fax: +86 20 83062807; E-mail: bbmcwei@126.com (WW); hejx@vip.163.com (JXH)

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