

# Prognostic value of D-lactate dehydrogenase in patients with clear cell renal cell carcinoma

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Received September 1, 2016; Accepted September 28, 2017

DOI: 10.3892/ol.2018.8782

**Abstract.** Lactate dehydrogenase (LDH) is an enzyme involved in anaerobic glycolysis and is associated with the prognosis of patients with renal cell carcinoma (RCC). The human genome has four LDH genes: *LDHA*, *LDHB*, *LDHC* and *LDHD*. In order to determine which of these four LDH genes may predict clear cell RCC (ccRCC), a total of 509 patients with ccRCC from The Cancer Genome Atlas (TCGA) cohort and 192 patients with ccRCC from the Fudan University Shanghai Cancer Centre (FUSCC) cohort were enrolled in the present study. The expression profiles of *LDHD* genes in the TCGA cohort were obtained from the TCGA RNAseq database. The Cox proportional hazards regression model and Kaplan-Meier curves were used to assess relative factors. The LDH family genes that were revealed to have an association with overall survival (OS) were further validated in the FUSCC cohort. In the TCGA cohort, following Cox proportional hazards analysis, *LDHD* expression ( $P=0.0400$ ; hazard ratio, 0.872; 95% confidence interval, 0.764-0.994) was revealed to be predictive of the prognosis of patients with ccRCC. Further analysis revealed that low *LDHD* expression ( $P<0.0001$ ) was significantly associated with a poor prognosis in terms of OS. Additionally, the expression of *LDHD* ( $P<0.0001$ ) was significantly different in patients with ccRCC compared with paired controls. In the FUSCC cohort, low *LDHD* expression was also associated with a poor OS ( $P=0.0103$ ), and the tumour pathological T stage was a factor that influenced the expression of *LDHD* ( $P=0.0120$ ). Furthermore, the expression of *LDHD* influenced the serum LDH level ( $P=0.0126$ ). The

downregulation of *LDHD* expression may be a predictor of poor prognosis in patients with ccRCC.

## Introduction

Renal cancer is a metabolic disease that starts in the cells of the kidney (1) and is responsible for ~3% of all malignancies in adults (2). Globally, 250,000 new cases of kidney cancer are diagnosed each year (3). Renal cell carcinoma (RCC) accounts for ~90% of all renal cancer cases (4,5), and is the most common form of adult kidney cancer, possessing unique genetic and histological features. Radical nephrectomy (RN) is effective in curing early and local RCC, however, one-third of all patients present with metastatic disease at diagnosis (6). Furthermore, 20-40% of patients with RCC who undergo surgical nephrectomy will develop metastasis. Despite previous developments in therapy, there remains no effective treatment for patients with advanced stages of the disease, and RCC is generally resistant to standard chemotherapy and radiotherapy. The 5-year survival rate of patients with metastatic RCC is <10% (1,7). Important prognostic factors of RCC include histological subtype, nuclear grade, tumour size and evidence of metastatic disease at presentation (8). In order to further our understanding on the prognosis of RCC and in order to develop novel biological therapeutic methods, the identification of molecular markers, which are useful in assisting in the management of the patients, and novel molecular targets for adjuvant therapies, is necessary.

Lactate dehydrogenase (LDH) is a tetrameric enzyme, belonging to the 2-hydroxy acid oxidoreductase family (9); it is a metabolic enzyme that catalyses the interconversion of pyruvate and lactate during the processes of glycolysis and gluconeogenesis depending on nutrient availability. Previous studies have revealed that pre-treatment serum LDH is a statistically significant prognostic factor in breast, renal, lung and gastric cancer (10-13). LDH is under the translational control of the hypoxia-inducible factor and MYC proto-oncogene, BHLH transcription factor (MYC), and is thus regulated by key oncogenic processes. Previous studies have revealed that pre-treatment serum LDH is a significant prognostic factor in high-risk patients with metastatic RCC (14,15). In addition, the activation of these oncogenic pathways resulted in high serum LDH levels, which were associated with drug resistance (14).

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**Key words:** clear cell renal cell carcinoma, D-lactate dehydrogenase, prognosis

The human genome contains four LDH genes: *LDHA*, *LDHB*, *LDHC* and *LDHD* (16). The LDH family may increase the rate of the simultaneous interconversion of pyruvate to lactate and nicotinamide adenine dinucleotide (NAD)H to NAD<sup>+</sup> by 14 orders of magnitude (17,18). Numerous genes and proteins associated with apoptosis or tumour survival have been reported to be associated with LDH activity (19,20). However, the majority of previous studies on renal cancer focused on serum LDH, and few studies have analysed specifically which LDH gene serves a key function in RCC.

## Materials and methods

**LDH expression data.** LDH expression and clinical data from The Cancer Genome Atlas (TCGA) database were sourced from the Cancer Genomics Browser of University of California Santa Cruz (<https://genome-cancer.ucsc.edu/>). A total of 6 members of the LDH family are included in the database, including *LDHA*, *LDHB*, *LDHC*, *LDHD*, LDH A like 6A (*LDHAL6A*) and *LDHAL6B*. In total, 509 primary clear cell RCC (ccRCC) tumours from patients with detailed LDH expression data obtained between January 1998 and December 2013 were selected from the updated TCGA database according to parameters defined in a previous study (21). Patients with fully characterized tumours, intact overall survival (OS) data, complete RNAseq information and those without pre-treatment were included. Data on clinicopathological characteristics, including age, sex, tumour size, Tumour-Node-Metastasis (TNM) stage (22), Fuhrman grade (23), AJCC renal cancer stage (24), laterality, haemoglobin level, white blood cell count, platelet level and OS time were collected. A follow-up of the patients was completed, with a median length of 1,063 days. During the follow-up, 347 patients succumbed.

**Patient enrollment.** From the Fudan University Shanghai Cancer Centre (FUSCC) cohort, a total of 192 patients with ccRCC who underwent RN or nephron-sparing nephrectomy between 2007 and 2011 were retrospectively enrolled. All the tissue samples were collected during surgery and stored at -70°C in the tissue bank of the FUSCC. The pathological subtypes were determined by two pathologists, who were genitourinary specialists. Data on the clinicopathological characteristics, including sex and tumour size, were collected.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RNA extraction, RT and RT-qPCR analysis were performed on the FUSCC cohort. In the FUSCC cohort, total RNA was isolated from the 192 ccRCC samples using TRIzol<sup>®</sup> reagent (cat no. 15596-026; Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). A PrimeScript RT reagent kit (cat no. K1622; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's protocol to synthesize first-strand cDNA from 1 µg total RNA isolated from renal carcinoma cells. Serial dilutions of cDNA were amplified by qPCR using gene-specific primers. The most concentrated sample contained cDNA derived from 1 ng total RNA. Next, SYBR Green Real-Time PCR assays (Thermo Fisher Scientific, Inc.) were performed using an ABI 7900HT (Applied Biosystems, Thermo Fisher Scientific, Inc.). The

expression level of RNA was normalized to the level of β-actin. The primers for RT-qPCR analysis were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences used were as follows: *LDHD* forward, 5'-CAAAGCCAGGGA GGGGAAGAG-3' and reverse, 5'-CGTAGTCAGGGA ACT TGTGGG-3'; *LDHAL6B* forward, 5'-TTCCGAGAAGCC CGTTCATC-3' and reverse, 5'-GTGAAAGGGCTGCCATGT TG-3'. And β-actin forward, 5'-AGCGAGCATCCCCAAAG TT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3'. The efficiency of amplification of the target gene (*LDH family members*) and the internal control (β-actin) was examined using qPCR and TaqMan detection. Thermocycling conditions were as follows: i) Initial denaturation at 94°C for 4 min; ii) 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 25 sec and extension at 72°C for 45 sec; and iii) a final extension at 72°C for 5 min (25). Quantification was performed using the 2<sup>-ΔΔC<sub>q</sub></sup> method (25). TNM, tumor grade, tumor stage, serum LDH level and tumor position were obtained from electronic records. Patients received regular follow-ups via telephone call or in the clinic once every 3 months. Events, including tumor recurrence, progression, metastasis and mortality were recorded.

**Statistical analysis.** The disease-free survival time was calculated from the date of diagnosis until the date of mortality from any cause or first recurrence. The OS time was defined as the time of diagnosis until the date of the last follow-up or mortality from any cause. Patients without recurrence events or mortality were recorded as censored at the time of the last follow-up. Statistical analysis was performed using Stata software (version 12.0; StataCorp LP, College Station, TX, USA). All statistical tests were two-tailed, and P<0.05 was considered to indicate a statistically significant difference. Survival curves were constructed using Kaplan-Meier curves and a log-rank test in order to assess the differences between the groups. Adjusted odds ratios with 95% confidence intervals (CIs) were calculated using Cox proportional hazards models. Univariate and multivariate Cox proportional hazards analysis assessed the expression of LDH family members and OS for patients with ccRCC in the TCGA cohort. Genes known to be associated with OS were further studied. Multivariate logistic regression was performed in order to further study factors that may affect the expression of LDHs. Student's t-test or Wilcoxon's signed rank test were used for 70 paired patients in order to reveal potential differences in the expression of LDH family members between patients with ccRCC and a control population. Spearman's correlation analysis was used to determine the correlation between LDH levels and the expression of *LDHD*.

## Results

**Clinical characteristics of patients with ccRCC.** In the TCGA cohort, the median age of the 509 patients with ccRCC was 61 years (range, 26-90 years). A total of 328 (64.4%) patients were male and 181 (35.6%) patients were female. The median follow-up time of this cohort was 35.4 months. TNM, tumor size, tumor grade, tumor stage, laterality, white blood cell count, hemoglobin level and platelet level are presented in Table I.

Table I. Clinicopathological characteristics of patients in the TCGA and FUSCC cohorts.

Variables	TCGA cohort	FUSCC cohort
Total number of patients, n (%)	509	192
Median age (range), years	61 (26-90)	55.5 (17-84)
Sex, n (%)		
Male	328 (64.44)	131 (68.23)
Female	181 (35.56)	61 (31.77)
Fuhrman grade, n (%)		
1 and 2	234 (45.97)	79 (41.15)
3 and 4	271 (53.24)	113 (58.85)
Gx	4 (0.79)	0 (0.00)
Mean longest dimension (range), cm	5.30 (0-20.0)	5.01 (1-16.0)
T, n (%)		
T1	258 (50.69)	129 (67.19)
T2	63 (12.38)	29 (15.10)
T3	178 (34.97)	27 (14.06)
T4	10 (1.96)	7 (3.64)
N, n (%)		
N0	228 (44.79)	181 (94.27)
N1	18 (3.54)	4 (2.08)
Nx	263 (51.67)	7 (3.64)
M, n (%)		
M0	406 (79.76)	184 (95.80)
M1	78 (15.32)	7 (3.60)
Mx	25 (4.91)	1 (0.50)
Stage, n (%)		
I	253 (49.71)	130 (67.71)
II	51 (10.02)	30 (15.62)
III	125 (24.56)	23 (11.98)
IV	80 (15.72)	9 (4.69)
Laterality, n (%)		
Left	239 (46.95)	90 (46.87)
Right	269 (52.85)	94 (48.95)
Bilateral	1 (0.20)	8 (41.67)
Median hemoglobin (range), g/l		139 (76-398)
Elevated, n (%)	5 (0.98)	20 (10.42)
Normal, n (%)	175 (34.38)	86 (44.79)
Low, n (%)	251 (49.31)	16 (8.33)
Undefined, n (%)	78 (15.32)	70 (36.46)
Median white blood cell count (range), 10 <sup>9</sup> /l		6.2 (3.1-18.4)
Elevated, n (%)	160 (31.43)	3 (1.56)
Normal, n (%)	251 (49.31)	100 (52.08)
Low, n (%)	8 (1.57)	17 (8.85)
Undefined, n (%)	89 (17.49)	72 (37.5)
Median platelet level (range), 10 <sup>9</sup> /l		219 (97-450)
Elevated, n (%)	35 (6.88)	4 (2.08)
Normal, n (%)	342 (67.19)	103 (53.65)
Low, n (%)	45 (8.84)	2 (1.04)
Undefined, n (%)	86 (16.90)	83 (43.23)
Median follow-up time (range), days	1,063 (2-3,668)	1,412 (117-2,245)
Median serum LDH level (range), U/l <sup>a</sup>		155 (67-761)

<sup>a</sup>No data present in the TCGA database. TCGA, The Cancer Genome Atlas; FUSCC, Fudan University Shanghai Cancer Centre; Gx, grade cannot be assessed; T, tumour; N, node; M, metastasis; LDH, lactate dehydrogenase.

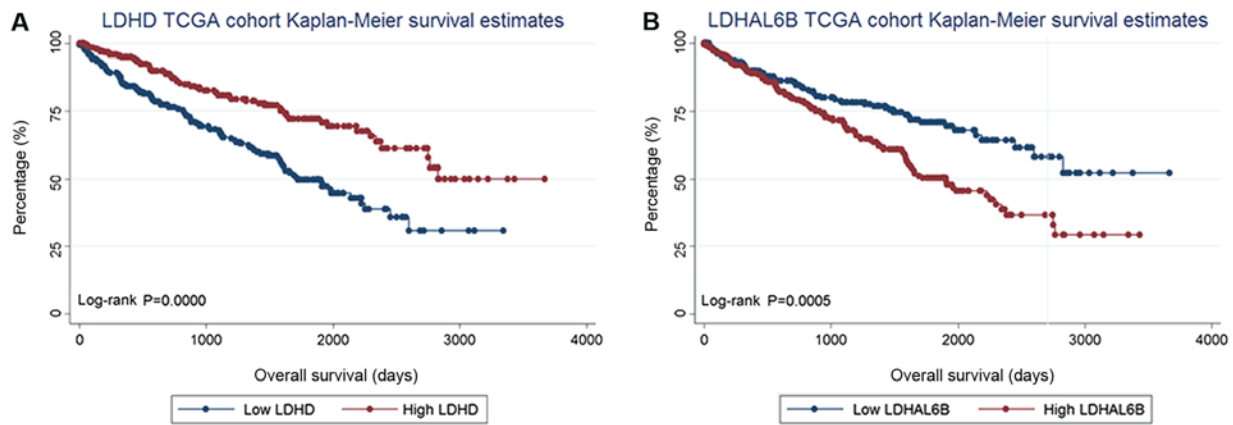


Figure 1. Kaplan-Meier plots of survival in the TCGA cohort are presented according to *LDHD* and *LDHAL6B* expression. (A) Kaplan-Meier estimates of OS are presented according to the expression of *LDHD*. (B) Kaplan-Meier estimates of OS are presented according to the expression of *LDHAL6B*. TCGA, The Cancer Genome Atlas; LDH, lactate dehydrogenase; *LDHAL6B*, LDH A like 6B; OS, overall survival.

In the FUSCC cohort, the median age of the 192 patients with ccRCC was 55.5 years (range, 17-84 years). A total of 47 patients succumbed during the follow-up, and the median follow-up time of this cohort was 47.1 months. A total of 131 (68.2%) of patients were male and 61 (31.8%) of patients were female. TNM, tumor size, tumor grade, tumor stage and laterality are additionally presented in Table I.

*LDHD* and *LDHAL6B* are independent prognostic factors for OS. In the TCGA cohort, a univariate Cox proportional hazards model was performed to assess the factors predicting OS. Age, TNM stage, Fuhrman grade, haemoglobin level, white blood cell (WBC) count, platelet (PLT) count, and *LDHC*, *LDHD* and *LDHAL6B* expression were significantly associated with the OS of the patients with ccRCC (all  $P < 0.05$ ; Table II). A multivariate Cox analysis performed following adjustment for all the potential prognostic factors, which included age, tumor stage, Fuhrman grade, laterality, WBC count, PLT count, hemoglobin content, *LDHD*, *LDHC* and *LDHAL6B* expression indicated that age (HR, 1.036; 95% CI, 1.020-1.053;  $P < 0.0001$ ), tumor stage (HR, 1.603; 95% CI, 1.317-1.949;  $P < 0.0001$ ), laterality (HR, 0.664; 95% CI, 0.467-0.944;  $P = 0.023$ ), *LDHD* expression (HR, 0.872; 95% CI, 0.764-0.994;  $P = 0.040$ ) and *LDHAL6B* expression (HR, 1.285; 95% CI, 1.048-1.576;  $P = 0.016$ ) were the only independent predictors of OS (all  $P < 0.05$ , Table II).

Expression quantities of *LDHD* and *LDHAL6B* are associated with prognosis and OS. As *LDHD* and *LDHAL6B* expression were the only independent predictors of OS in multivariate Cox analysis, they were selected for analysis. *LDHD* expression and *LDHAL6B* expression were revealed to be normally distributed, and were thus considered as categorical variables according to the median expression level (divided into low and high expression groups according to the median values of *LDHD* and *LDHAL6B* of 7.24 and 1.49, respectively). As a result, it was revealed that low *LDHD* expression ( $P < 0.0001$ ) was associated with a poor prognosis for OS, whereas a low level of *LDHAL6B* expression ( $P = 0.0005$ ) was associated with an improved prognosis for OS, compared with their low expression counterparts (Fig. 1). *LDHD* and *LDHAL6B* expression

were considered to be categorical variables according to the median expression level. A log-rank test was performed in order to compare the survival curves between the different serum LDH levels.

In order to further understand the factors that may affect the expression of *LDHD* and *LDHAL6B*, a multivariate logistic regression analysis was performed. Tumor pathological T stage was revealed to be significantly associated with *LDHD* ( $P = 0.003$ ), whereas haemoglobin ( $P = 0.003$ ) was significantly associated with *LDHAL6B* expression (Table III).

The expression of LDH members in 70 paired patients from the TCGA database was then analysed to understand the difference in the expression of LDH family members between patients with ccRCC and a control group. A paired Student's t-test was used if the deviations of LDH expression between couples fitted a normal distribution, and the Wilcoxon signed rank test was employed for those that did not fit a normal distribution. In the paired Student's t-tests, the expression of *LDHD* ( $P < 0.0001$ ) and *LDHAL6A* ( $P < 0.0001$ ) were significantly different between the patients with ccRCC and the paired controls, whereas the expression of *LDHAL6B* ( $P = 0.375$ ) was not significantly different. In the Wilcoxon's signed rank test analysis, the expression of *LDHA* ( $P < 0.0001$ ), *LDHB* ( $P < 0.0001$ ) and *LDHC* ( $P < 0.0001$ ) was significantly different between patients with ccRCC and the paired controls (Table IV).

*LDHD* expression is a prognostic factor for OS in the FUSCC cohort. In the FUSCC cohort, *LDHD* and *LDHAL6B* expression was validated. The expression of *LDHD* and *LDHAL6B* was considered as categorical variables according to the median expression level (low and high expression groups). As the expression level of genes was based on the relative values of the PCR results, patients were grouped by  $\Delta\text{-Cq}$  (cycle threshold).  $\Delta\text{-Cq} = \text{Cq}(\text{target genes}) - \text{Cq}(\text{reference genes})$ . The median  $\Delta\text{-Cq}$  values of *LDHD* and *LDHAL6B* were 5.93 and 1.77, respectively. As a result, low *LDHD* expression was associated with a poor prognosis for OS (log-rank test,  $P = 0.010$ ), whereas the expression of *LDHAL6B* (log-rank test,  $P = 0.412$ ) was not associated with OS. The Kaplan-Meier curves are presented in Fig. 2.

Table II. Univariate and multivariate Cox proportional hazard analysis of *LDHD* expression and overall survival for patients with clear cell renal cell carcinoma in the TCGA and FUSCC cohort.

Variables	Univariate (TCGA)			Multivariate (TCGA)			Univariate (FUSCC)			Multivariate (FUSCC)		
	HR	(95% CI)	P-value	HR	(95% CI)	P-value	HR	(95% CI)	P-value	HR	(95% CI)	P-value
Age	1.028	(1.015-1.042)	<0.0001	1.036	(1.020-1.053)	<0.0001	1.001	(0.997-1.025)	0.932	0.996	(0.961-1.034)	0.858
Sex	1.073	(0.781-1.473)	0.665									
T	1.964	(1.658-2.325)	<0.0001									
N	2.799	(1.486-5.274)	0.001									
M	4.448	(3.221-6.141)	<0.0001									
AJCC stage	1.944	(1.695-2.229)	<0.0001	1.603	(1.317-1.949)	<0.0001	2.065	(1.597-2.671)	<0.0001	2.325	(1.538-3.514)	<0.0001
Fuhrman grade	2.350	(1.899-2.908)	<0.0001	1.230	(0.920-1.644)	0.162	2.007	(1.309-3.077)	0.001	0.959	(0.529-1.738)	0.891
Hb	0.584	(0.415-0.823)	0.002	0.915	(0.624-1.342)	0.651	1.003	(0.995-1.011)	0.534	1.008	(0.985-1.032)	0.493
WBC	0.652	(0.471-0.902)	0.010	1.014	(0.694-1.483)	0.942	0.994	(0.986-1.002)	0.142	0.985	(0.972-1.000)	0.043
PLT	1.702	(1.145-2.529)	0.008	1.086	(0.748-1.579)	0.664	1.004	(1.000-1.008)	0.061	1.005	(0.999-1.010)	0.083
Tumour size	1.174	(0.946-1.459)	0.146									
Laterality	0.669	(0.491-0.913)	0.011	0.664	(0.467-0.944)	0.023	1.075	(0.606-1.909)	0.803	1.197	(0.544-2.635)	0.655
<i>LDHA</i>	0.894	(0.690-1.158)	0.396									
<i>LDHAL6B</i>	1.552	(1.286-1.874)	<0.0001	1.285	(1.048-1.576)	0.016	1.010	(0.963-1.059)	0.680	1.066	(0.994-1.144)	0.073
<i>LDHAL6A</i>	0.895	(0.752-1.065)	0.212									
<i>LDHB</i>	1.087	(0.813-1.453)	0.574									
<i>LDHC</i>	1.093	(1.002-1.193)	0.046	1.073	(0.978-1.177)	0.137						
<i>LDHD</i>	0.765	(0.682-0.858)	<0.0001	0.872	(0.764-0.994)	0.040	0.944	(0.897-0.994)	0.029	0.899	(0.826-0.979)	0.014

LDH, lactate dehydrogenase; LDHAL6B, LDH A like 6B; TGCA, The Cancer Genome Atlas; FUSCC, Fudan University Shanghai Cancer Centre; HR, hazard ratio; CI, confidence interval; T, tumor; N, node; M, metastasis; Hb, hemoglobin; WBC, white blood cell; PLT, platelet.



Table III. Multivariate logistic regression analysis of factors that may affect the expression of *LDHD* and *LDHAL6B* in The Cancer Genome Atlas cohort with clear cell renal cell carcinoma.

Variables	<i>LDHD</i>			<i>LDHAL6B</i>		
	OR	(95% CI)	P-value	OR	(95% CI)	P-value
Age	1.013	(0.987-1.040)	0.314	1.0114	(0.986-1.038)	0.382
T	0.565	(0.389-0.819)	0.003	1.0560	(0.734-1.520)	0.771
N	0.965	(0.255-3.650)	0.958	0.7600	(0.216-2.671)	0.669
M	1.652	(0.695-3.926)	0.256	0.6730	(0.286-1.581)	0.363
Fuhrman grade	0.788	(0.499-1.243)	0.305	1.0480	(0.665-1.649)	0.841
Hb	1.320	(0.700-2.488)	0.391	0.3800	(0.202-0.717)	0.003
WBC	1.048	(0.564-1.948)	0.881	0.6880	(0.371-1.276)	0.236
PLT	1.065	(0.564-2.011)	0.847	1.4330	(0.756-2.718)	0.271

LDH, lactate dehydrogenase; *LDHAL6B*, LDH A like 6B; OR, odds ratio; CI, confidence interval; T, tumor; N, node; M, metastasis; Hb, hemoglobin; WBC, white blood cell; PLT, platelet.

Table IV. Expression of LDH family members in 70 paired patients in The Cancer Genome Atlas cohort.

Variable	P-value	Statistical method	(95% CI)
<i>LDHA</i>	<0.0001	Wilcoxon's rank sum test	
<i>LDHAL6B</i>	0.3750	Paired Student's t-test	(-0.103-0.270)
<i>LDHAL6A</i>	<0.0001	Paired Student's t-test	(0.689-1.227)
<i>LDHB</i>	<0.0001	Wilcoxon's rank sum test	
<i>LDHC</i>	<0.0001	Wilcoxon's rank sum test	
<i>LDHD</i>	<0.0001	Paired Student's t-test	(2.269-3.168)

LDH, lactate dehydrogenase; *LDHAL6B*, LDH A like 6B; CI, confidence interval.

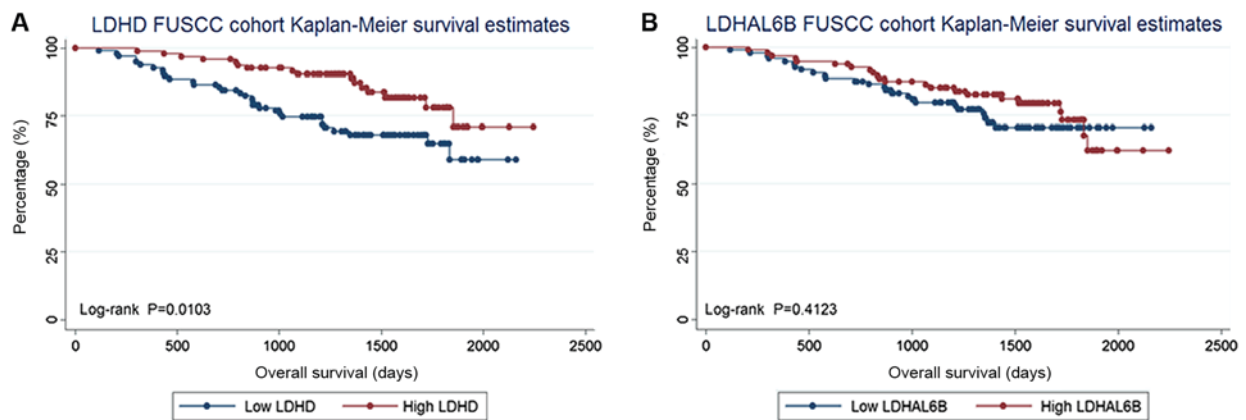


Figure 2. Kaplan-Meier plots of survival in the FUSCC cohort are presented according to *LDHD* and *LDHAL6B*. (A) Kaplan-Meier estimates of OS are presented according to the expression of *LDHD*. (B) Kaplan-Meier estimates of OS are presented according to the expression of *LDHAL6B*. FUSCC, Fudan University Shanghai Cancer Centre; LDH, lactate dehydrogenase; *LDHAL6B*, LDH A like 6B; OS, overall survival.

In order to further understand the factors that may affect the expression of *LDHD* and *LDHAL6B* in the FUSCC cohort, a multivariate logistic regression analysis using the same parameters was performed. It was revealed that in the FUSCC cohort, tumor pathological T stage was significantly associated with the expression of *LDHD* ( $P=0.012$ ), whereas age

was significantly associated with the expression of *LDHAL6B* ( $P=0.043$ ; Table V).

*Expression level of LDHD influences the serum LDH level.* As there was no data concerning the serum LDH level in the TCGA database, the present study tested the serum LDH of patients

Table V. Multivariate logistic regression analysis of factors that may affect the expression of *LDHD* and *LDHAL6B* in the FUSCC cohort with ccRCC.

Variables	<i>LDHD</i>			<i>LDHAL6B</i>		
	OR	(95% CI)	P-value	OR	(95% CI)	P-value
Age	0.999	(0.962-1.038)	0.971	0.962	(0.926-0.999)	0.043
T	0.519	(0.312-0.864)	0.012	0.959	(0.604-1.523)	0.859
N	11.272	(0.856-148.417)	0.065	2.651	(0.223-31.479)	0.440
M	1.056	(0.069-16.042)	0.969	0.521	(0.6038-7.080)	0.624
Fuhrman grade	0.587	(0.295-1.169)	0.130	0.579	(0.300-1.117)	0.103
Hb	0.986	(0.958-1.013)	0.305	1.000	(0.974-1.026)	0.978
WBC	0.997	(0.988-1.007)	0.578	0.995	(0.984-1.006)	0.373
PLT	1.003	(0.997-1.009)	0.300	1.001	(0.995-1.007)	0.733

LDH, lactate dehydrogenase; *LDHAL6B*, LDH A like 6B; FUSCC, Fudan University Shanghai Cancer Centre; OR, odds ratio; CI, confidence interval; T, tumor; N, node; M, metastasis; Hb, hemoglobin; WBC, white blood cell; PLT, platelet.

from the FUSCC cohort when they were diagnosed with a kidney tumor in order to understand whether the expression of *LDHD* is associated with serum LDH. In this further analysis of the FUSCC cohort, the 192 patients were divided into two groups according to their serum LDH level. Log-rank testing revealed that a serum LDH level higher than the upper limit of normal (215 U/l) was associated with a poor prognosis for OS (log-rank test,  $P=0.006$ ; Fig. 3). This result is concurrent with those of previous studies (11,14,21,26). Furthermore, Spearman's correlation analysis (data not shown) revealed that the serum LDH level has a negative correlation with the expression of *LDHD* ( $P=0.028$ ). Despite the fact that the analysis of serum LDH is a protein test and the analysis of *LDHD* is a gene test, it may be concluded that the expression of *LDHD* is associated with the levels of serum LDH.

*Expression of LDHD and LDHAL6B is not associated with recurrence-free survival (RFS) and cancer-specific survival (CSS) in the FUSCC cohort.* In order to understand if the expression of *LDHD* and *LDHAL6B* were associated with RFS and CSS, *LDHD* and *LDHAL6B* expression in the FUSCC cohort was considered as a categorical variable according to the median expression level, and divided into high and low expression groups as aforementioned. RFS was measured from the date of renal resection until the date of radiographic detection of recurrence or the last follow-up. CSS was measured from the date of renal resection until the the date of mortality due to renal cancer or the last follow-up. A total of 18 patients were diagnosed with radiographic recurrence and 10 patients succumbed to renal cancer during the follow-up. As a result, it was revealed that the expression of *LDHD* was not associated with RFS (log-rank test,  $P=0.887$ ) or CSS (log-rank test,  $P=0.133$ ). *LDHAL6B* expression was also not associated with RFS (log-rank test,  $P=0.364$ ) and CSS (log rank test,  $P=0.430$ ) (data not shown).

## Discussion

In the present study, it was demonstrated that certain LDH gene family members were associated with the OS of patients

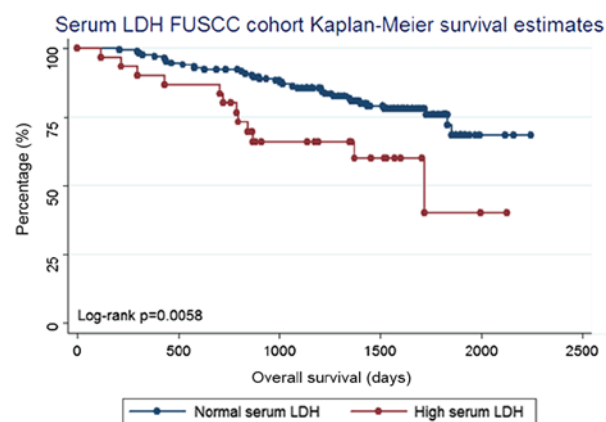


Figure 3. Kaplan-Meier plots of overall survival in the FUSCC cohort are presented according to serum LDH. FUSCC, Fudan University Shanghai Cancer Centre; LDH, lactate dehydrogenase.

with ccRCC. Members of this family, particularly *LDHD* and *LDHAL6B*, were independent prognostic factors for the OS of patients with ccRCC in the present study.

The human genome has four LDH genes: *LDHA*, *LDHB*, *LDHC*, and *LDHD*. Of these genes (26), *LDHB* and *LDHC* are L isomers and *LDHD* is a D isomer (27).

*LDHD* metabolism has been demonstrated to occur in mitochondria, but its function in cancer is unclear. Previous studies have demonstrated that in prostate cancer cells, the metabolism of D-lactate inside mitochondria via *LDHD* and the metabolic activity of *LDHD* in tumor cells were higher compared with that in normal cells (28,29). Furthermore, the *LDHD* protein level and activity are higher in cancerous cells compared with that in normal prostate cells (30). *LDHD* is a unique gene; in *Sporolactobacillus inulinus* strain CASD, *LDHD* may use NADH and NADPH, but preferentially uses NADPH as a coenzyme, which is different from the coenzyme utilization of other LDHs (31,32).

A feature of tumor cells is their reliance upon fermentative glycolysis, a common phenomenon coined 'the Warburg

effect' (33). LDH isoenzymes are metabolically regulated, and are linked to glycolysis and the Warburg effect (34). Previous studies have revealed that the level of pre-treatment serum LDH is a significant prognostic factor in numerous types of cancer, including RCC (35-37). LDH is under the translational control of hypoxia-inducible factor and MYC, and is thus regulated by key oncogenic processes (33,38). However, in the field of oncology, little is known concerning the subtypes of the LDH family. The few previous studies focused on this topic have been mainly confined to the cellular level. RCC is a disease that is closely associated with metabolism (39,40). The LDH gene family serves an important function in ccRCC. *LDHA* may possess multiple additional functions in non-neoplastic and neoplastic tissues (41). There are numerous genes and proteins, including tumor protein p53, vascular endothelial growth factor and 2'-deoxynucleoside 5'-phosphate N-hydrolase 1, amongst others, reported to be associated with *LDHA* activity (41). The expression of *LDHA* is associated with the prognosis of patients with brain tumors. A previous study revealed that *LDHA* metabolic activity in brain tumor cells was stronger compared with normal cells (41). However, the clinical association between *LDHD* expression and tumors is not clear. There are also few studies focused on renal cancer.

The present study revealed that *LDHD* may serve an important function in the prognosis of patients with ccRCC. It was revealed that the expression of *LDHD* and *LDHAL6B* were independent prognostic factors for the OS of patients with ccRCC. The downregulated expression of *LDHD* was associated with a poor prognosis and a shorter OS. The expression of *LDHD* is associated with the prognosis of patients with ccRCC. In addition, the tumor stage was significantly associated with the expression of *LDHD*. In the statistical analysis of 70 paired patients, the expression of *LDHD* was also significantly different between patients with ccRCC and paired controls. Furthermore, the serum LDH level was significantly associated with the expression of *LDHD*. The results of the present study confirmed that *LDHD* is a useful biomarker for patients with ccRCC. In addition, the expression of *LDHD* was associated with patient prognosis. To the best of our knowledge, no previous study has clarified the function of *LDHD* in renal tumors. Although Girgis *et al* (42) had previously assessed the expression of *LDHA* at the protein level via the use of immunohistochemistry in 385 patients and indicated that *LDHA* upregulation may be a predictor of a poor prognosis in patients with ccRCC, in the present study, *LDHA* expression was not significantly associated with the OS of patients with ccRCC in the TCGA cohort or the FUSCC cohort.

*LDHD* is a subtype of LDH and serves an important function in renal cancer. The integration of *LDHD* expression has the potential to be a useful biomarker in the identification of poor prognosis in patients with ccRCC. Although it has potential as a novel renal cancer biomarker, its specific mechanism remains unknown. To date, *LDHD*, which is produced in the methylglyoxal (MG) pathway, is presumed to be released by cancer cells (43). The MG pathway produces *LDHD* as a final product that is largely modified in cancer cells, with a specific function in the glyoxalase systems, serving to eliminate the cytotoxic MG mainly derived from glycolysis (44). The rationally engineered *LDHD* may efficiently use NADH and NADPH as cofactors. Additionally,

the mitochondrial metabolism of *LDHD* in cancer cells is more active compared with that of normal cells (45). Renal cancer is a disease that is associated with metabolism and obesity. *LDHD* may serve a key function in the progression and development of ccRCC.

There are a number of limitations to the present study: Firstly, the patients included were from the FUSCC, with good follow up, but patients from other centres were not included. Secondly, all the tissue specimens in the present study were sourced from patients who were suitable candidates for surgery, and the same results may not apply to patients who are not suitable candidates for surgery.

The present study has indicated at the association between ccRCC outcome and *LDHD*, however, the underlying mechanism remains poorly understood. The present study may have opened a threshold to a novel aspect of ccRCC biomarkers or therapeutic targets. Further studies are required.

In conclusion, *LDHD* was identified as an independent prognostic factor for the OS of patients with ccRCC. Low *LDHD* expression was associated with a poor prognosis for OS, and tumor grade was significantly associated with *LDHD* expression. *LDHD* may function as a tool to reveal further genes associated with prognosis in ccRCC.

#### Acknowledgements

The present study was supported by the International Cooperation and Exchange of Science and Technology Commission of the Shanghai Municipality (grant no. 12410709300), the Guide Project of Science and Technology Commission of Shanghai Municipality (grant no. 124119a7300), and the Outstanding Young Talent Training Plan of Shanghai Municipal Commission of Health and Family Planning (grant no. XYQ2013102).

#### Competing interests

The authors declare that they have no competing interests.

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