Serum glycine dehydrogenase is associated with increased risk of lung cancer and promotes malignant transformation by regulating DNA methyltransferases expression

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Abstract. Identification of novel risk factors that are critical to the initiation of lung cancer will be key for its prevention. Recently, it has been reported that glycine dehydrogenase (GLDC) can drive the formation of lung cancer initiating cells. However, there have been no perspective studies on the association between circulating GLDC and lung cancer until now. To identify whether serum GLDC is a risk factor for lung cancer, the present study conducted a nested case-control study within a Chinese cohort. Using ELISAs, serum GLDC was measured in 300 case subjects, who were subsequently diagnosed with lung cancer during follow-up, and in 600 matched healthy controls. The results revealed that serum GLDC was associated with increased lung cancer risk [odds ratio=1.48; 95% confidence intervals (1.01-2.04)]. Spearman correlation was employed to analyze the associations between age, body mass index, years of smoking and the serum concentration of GLDC. It was demonstrated that years of smoking was associated with serum GLDC (spearman's correlation, ϱ=0.81) in patients with lung cancer. However, the association was attenuated in the serum of matched controls (ϱ=0.48). In addition, overexpression of GLDC protein contributed to malignant transformation and inhibited microRNA (miR)-29 family expression in normal human bronchial epithelial (NHBE) cells. Aberrant methylation of tumor suppressive gene (TSG) is an early event in the development of lung cancer, which is controlled by DNA methyltransferases (DNMTs). The present study demonstrated that GLDC promoted the expression of DNMT proteins; however, the miR-29 family inhibited their expression in NHBE cells. Thus, it was concluded that elevated serum GLDC may increase lung cancer risk, and that smoking, GLDC, the miR-29 family and DNMT signaling pathways may serve an important role in early malignant transformation during the development of lung cancer.

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Abbreviations: TSG, tumor suppressive gene; GLDC, glycine dehydrogenase; NSCLC, non-small cell lung cancer

Key words: lung cancer, glycine dehydrogenase, microRNA-29a/b/c, DNA methyltransferases

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1). Identifying novel risk factors is essential in order to prevent the disease. Abnormal overexpression of the metabolic enzyme glycine dehydrogenase (GLDC) has been associated with lung cancer and other types of tumors (2-4). GLDC is critical for the formation of cancer initiating cells in non-small cell lung cancer (NSCLC) (2). In animal models, its overexpression can induce malignant transformation of lung normal cells and promote the formation of tumors (2). However, there is currently no perspective study on the associations between GLDC and lung cancer.

DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring, which is a hallmark of human diseases such as lung cancer (5-7). Methylation of DNA at position 5 of the cytosine ring is catalyzed by DNA methyltransferases (DNMTs) and is the predominant epigenetic modification in mammals (8). The mammalian DNMT family includes 4 active members: DNMT1, DNMT3A, DNMT3B and DNMT3L (9,10). DNMT1 is the most abundant DNMT and is involved in the maintenance of methylation (8,11,12). DNMT3 functions as a *de novo* methyltransferase and consists of 2 associated proteins encoded by the distinct genes, DNMT3A and DNMT3B (11). The expression levels of these DNMTs are reportedly elevated in cancers of the colon, prostate, breast, liver and in leukemia (13-16). Aberrant methylation of the tumor suppressive gene (TSG) is an early event in the development of lung cancer (17,18). MicroRNAs (miRNA/miRs) are a group of small non-coding RNAs (~22 nucleotides) that regulate gene expression (19-21). The expressions of miR-29a, -29b and -29c were downregulated in NSCLCs (7). Expression of the miR-29 family is inversely associated with DNMTs expression in lung cancer tissues and the miR-29 family directly targets

DNMTs; the miR-29 family can revert aberrant methylation in lung cancer by targeting DNMTs (7). In addition, the enforced expression of the miR-29 family in lung cancer cell lines restored normal patterns of DNA and promoted the re-expression of TSGs silenced by methylation (7). However, the regulatory mechanism associated with miR-29 family expression has not been fully elucidated. The aim of the study was to assess the association between serum GLDC and lung cancer risk and study the mechanism underlying the effects of GLDC in lung cancer.

Materials and methods

Study cohort and serum samples. A nested case-control study was conducted in the well-characterized Chinese Cohort (22). The project included 300 invasive lung cancer cases, each of which were matched with 2 controls (n=600). The participants were recruited in Shandong Cancer Hospital and Shanghai cancer institute between 1998 and 2013, when they received physical examination and the physical characteristics are presented in Table I. Each participant donated more than one blood sample at the recruitment. For each case-subject match set, 2 control subjects closest to the case (based on matching criteria, age at time of sampling) with an available blood sample were chosen among the appropriate risk sets consisting of all cohort members alive and free of cancer at the time of diagnosis of the index case. Serum aliquots of 500 μl were stored at -180˚C for measurements of GLDC. The matching criterion was age at the time blood was drawn. The Shandong (China) Ethical Board of the Shandong Academy of Occupational Health and Occupational Medicine (Shandong, China) approved the present study and written informed consent was obtained from each individual recruited.

Cell culture. Normal human bronchial epithelial (NHBE) cells were obtained from the America Type Culture Collection (ATCC; Manassas, VA, USA) were grown in RPMI-1640 medium (Sigma, Shanghai, China) containing 10% fetal bovine serum (FBS; Shanghai ExCell Biology, China) and 100 mg/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere with 5% CO_2 (23).

Pre-miR-29a/-29b/29c/Control miR, GLDC expressing plasmids/empty vectors and transfection experiments. *Pre-miR-29a/-29b/29c/Control miR* were purchased from Ambion, (Thermo Fisher Scientific, Inc.). *GLDC* expressing plasmids and empty vectors (mock) were purchased from Tiangen (Beijing, China). For transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24 h, and then transfected with transfection reagent (Lipofectamine 2000; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. After incubation for 6 h, the medium was removed and replaced with normal culture medium for 48 h.

Enzyme-linked immunosorbent assay (ELISA). ELISA analysis was employed to detect the levels of serum GLDC protein in study cohort and was performed as described previously (24). The intra-batch and inter-batch coefficients of variation for GLDC protein were 4.54 and 8.73%, respectively. Multivariate unconditional logistic regression was performed to calculate the odds ratios (OR) and corresponding 95% confidence intervals (95% CI) for lung cancer occurrence, calculating ORs over the quartile levels and on a continuous $\log_2$ scale of circulating GLDC. The final multivariate models shown included 3 factors (years of smoking, asbestos exposure and sex) that affect the exposure and disease relation ≥10%. P_{trends} was calculated by using the median values of GLDC quartiles.

Western blot analysis. Western blot analysis was performed as described previously (25). For membrane incubation the following primary antibodies were used: Rabbit anti-GLDC (cat. no. ab232989; 1:500), anti-DNMT1 (cat. no. ab87654; 1:500) anti-DNMT3A (cat. no. EPR18455; 1:500), anti-DNMT3B (cat. no. ab2851; 1:500) and anti-β-actin (cat. no. ab5694; 1:500; all Abcam, Cambridge, MA, USA) antibodies for overnight incubation at 4˚C. Membranes were also incubated with IRDye™-800 conjugated anti-secondary antibodies (cat. no: ab6721; 1:10,000; Abcam) for 30 min at room temperature. For analysis, β-actin was a loading control. The specific proteins were visualized using the Odyssey™ Infrared Imaging System (Gene Company, Ltd., Hong Kong, China).

Colony formation assay. The colony formation assay was performed as described previously (26).

MTT assay. The effect on cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and was performed as described previously (27). Briefly, $1x10^4$ cells were seeded onto 96-well plates and were incubated for 24 h in a 37˚C, 5% CO_2 cell culture incubator. MTT reagent (50 μl; 5 mg/ml) was added to each well and cells were incubated for a further 4 h. Then the formazan precipitate was dissolved in 150 μl dimethylsulfoxide and the absorbance rate was measured in a microplate reader at a wave length of 570 nm, with the reference wavelength set at 630 nm. Absorbance was directly proportional to the number of surviving cells. The viability of the control group.

Microarray analysis of miRNA. Microarray Analysis of miRNA was performed as described previously (28).

Northern blotting analysis. Northern blot analysis of miRNAs was performed as described previously (29,30). The probe sequences were as follows: miR-29a, 5'-TAACCGATTTCA GATGGTGCTA-3'; miR-29b, 5'-AACACTGATTTCAAA TGGTGCTA-3'; miR-29c, 5'-TAACCGATTTCAAATGGT GCTA-3'; U6 small nuclear RNA, 5'-CCATGCTAATCTTCT CTGTATCGTTCCAA-3'.

Statistical analysis. The results of the colony formation and MTT assays were statistically analyzed, using a Student's t-test. Data were expressed as the mean ± standard error. Spearman correlation was performed for all other results in order to analyze the association between serum GLDC protein concentration and other variables (age, BMI and years of smoking). Multivariate logistic regression analysis was applied to assess

Table I. Baseline characteristics of the study cohort.

Characteristics	Mean		[a]P-value
	Cases (n=300)	Controls (n=600)	
Age, median (years)	60.1	59.2	
Sex (%)			
Men	82.7	80.2	
Women	17.3	19.8	0.65
Height (cm, median)	170.1	169.9	0.87
Weight (kg, median)	70.1	74.5	0.23
BMI (kg/m^2, median)	24.5	25.2	0.62
Smoking history			
No. of cigarette/day (median)	22.9	17.2	<0.01
Years of smoking (median)	32.9	27.3	<0.01
Asbestos exposure (%)	2.4	2.1	0.02
Daily dietary intake			
Total energy (kcal)	2,787	2,760	0.88
Carbohydrates (g, median)	285.4	297.3	0.09
Protein (g, median)	101.3	102.8	0.74
Saturated fat (g, median)	54.7	53.2	0.34
Education (%; >elementary school)	19.1	21.1	0.04
Physical activity (%; >3 h/week)	10.7	12.1	0.67

[a]P-values for nested case-control differences. BMI, body mass index.

Table II. Distribution of lung cancer by histological type.

Histological type	Number of cases (n=282)	Percentage of total (%)
Squamous cell carcinoma	62	22
Adenocarcinoma	94	33
Small cell carcinoma	40	14
Large/undifferentiated cell carcinoma	50	18
Other carcinoma or carcinoma unspecified	21	7
Unspecified morphology	15	5

Histological types were based on morphology codes grouped according to the World Health Organization scheme (20). Information for 18 of the 300 individuals enrolled in the present study was not available, therefore n=282.

the association between serum GLDC and lung cancer risk. All P-values presented are 2-sided and P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were conducted using SAS software, version 9.3 (SAS Institute, Inc., Cary, NC, USA).

Results

Higher serum GLDC is associated with increased risk in lung cancer. Baseline characteristics comparing 300 lung cancer cases and 600 matched controls are outlined in Table I; the matched variable was age. The median age was 60.1 years for lung cancer cases and 59.2 years for matched controls. The majority of the lung cancer cases and matched controls were men (82.7% for cases and 80.2% for controls). The cases had a lower average level of education, as well as a higher proportion of smokers (number of cigarettes per day or years of smoking) and greater occupational exposure to asbestos associated with lung cancer risk. However, no differences in body mass index (BMI) and physical activity were observed between the 2 groups. In addition, those with lung cancer had a higher energy intake rate as well as a lower consumption of fruit/carotenoids. In those with lung cancer, ~33% of cases were adenocarcinomas and 22% of the cases were squamous cell carcinomas (Table II). All other histological types of lung cancer accounted for <20% (Table II).

Serum GLDC was positively associated with the overall risk of lung cancer (Table III; OR=1.48; 95% CI, 1.01-2.04).

Table III. Adjusted odds ratios for lung cancer by quartile levels and on a continuous $\log_2$ scale of circulating prolactin (n=300).

	Quartile number					
Variable	1	2	3	4	Log_2 odds ratio (95% CI)	P_{trend}
Ca/Co	48/145	53/148	80/159	119/148	1.48 (1.01-2.04)	0.01
OR	1	0.99 (0.76,1.23)	1.15 (078,1.43)	1.59 (1.15-2.54)		

Values were adjusted for years of smoking, asbestos exposure and sex. OR values are presented as the OR (95% CI). OR, odds ratio; CI, confidence interval; Ca, Cancers; Co, Controls.

Table IV. Spearman's correlation coefficients between age, body mass index, years of smoking and serum glycine dehydrogenase in patients with lung cancer (n=300).

Variable	Age	BMI	Years of smoking
Glycine dehydrogenase	-0.28	0.08	0.81

BMI, body mass index.

Table V. Spearman's correlation coefficients between age, body mass, years of smoking and serum glycine dehydrogenase in matched controls (n=600).

Variable	Age	BMI	Years of smoking
Glycine dehydrogenase	-0.16	0.21	0.48

BMI, body mass index.

The risk was elevated in the highest quartile (OR=1.59; 95% CI, 1.15-2.54) when compared with the lowest quartile (OR=0.99; 95% CI, 0.76-1.23). Tables IV and V present the associations between age, BMI, years of smoking and GLDC in the lung cancer cases and the matched controls. Serum GLDC levels were positively correlated with years of smoking (Spearman's ϱ=0.81; Table IV); however, the association was attenuated in the sera of matched controls (Spearman's ϱ=0.48; Table V).

GLDC promotes tumorigenesis in normal human bronchial epithelial (NHBE) cells. To investigate whether GLDC promotes proliferation in NHBE cells, western blotting was performed to determine whether GLDC expressing plasmids can upregulate GLDC protein expression in NHBE cells. The results of western blotting showed that GLDC protein was upregulated by GLDC expressing plasmids in cells (Fig. 1A). To identify the role of GLDC in regulating proliferation, an MTT assay was performed. Overexpressing GLDC significantly promoted proliferation in NHBE cells (Fig. 1B). A colony formation assay was employed to detect whether GLDC protein affected the colony formation rate of the cells. The results demonstrated that GLDC promoted colony formation in NHBE cells (Fig. 1C).

GLDC inhibits miR-29a/b/c expression in NHBE cells. To examine the role of GLDC in the regulation of miRNA expression, microarray analysis was performed in NHBE cells. A total of 19 evidently altered miRNAs were identified; 9 miRNAs were downregulated and 10 miRNAs were upregulated with >5 fold changes in cells transfected with GLDC expressing plasmids compared with control cells (Table VI). Northern blot analysis was conducted to confirm whether overexpressing GLDC affected miR-29 a/b/c expression in NHBE cells. The results revealed that overexpressing GLDC markedly downregulated their expression in cells (Fig. 2).

Table VI. microRNA expression and glycine dehydrogenase regulation in human bronchial epithelial cells.

miRNA	Fold Change (GLDC vs. control)[a]	P-value
hsa-miR-29a	-105.34	$3.54\text{x}10^{-4}$
hsa-miR-29c	-75.07	$3.01\text{x}10^{-4}$
hsa-miR-29b	-63.04	$1.63\text{x}10^{-4}$
hsa-miR-1226	-32.85	$1.67\text{x}10^{-4}$
hsa-miR-146b-5p	-22.7	$4.57\text{x}10^{-4}$
has-miR-504	-12.55	$3.85\text{x}10^{-5}$
hsa-miR-98	-12.21	$7.63\text{x}10^{-5}$
hsa-miR-30b	-7.08	$2.45\text{x}10^{-5}$
has-miR-224	-6.05	$4.40\text{x}10^{-4}$
has-miR-297	5.68	$5.66\text{x}10^{-6}$
hsa-miR-1915-5p	5.84	$1.53\text{x}10^{-6}$
hsa-miR-183	15.25	$4.76\text{x}10^{-6}$
hsa-miR-1268	16.88	$3.68\text{x}10^{-4}$
hsa-miR-31	26.05	$3.70\text{x}10^{-5}$
hsa-miR-21	27.86	$4.10\text{x}10^{-6}$
hsa-miR-572	37.38	$1.09\text{x}10^{-5}$
has-miR-3188	41.89	$1.31\text{x}10^{-4}$
hsa-miR-1225-5p	43.14	$2.00\text{x}10^{-5}$
hsa-miR-328	46.30	$5.48\text{x}10^{-4}$

[a]Positive numbers indicate fold-induction and negative numbers indicate fold-repression. miRNA/miR, microRNA; GLDC, glycine dehydrogenase; hsa-, human.

GLDC promotes DNMTs protein expression and miR-29a/b/c inhibits their expression in NHBE cells. In order to detect

Table VII. Spearman's correlation coefficients between serum glycine dehydrogenase and microRNA-29 family in all participants (n=891[a]).

Variable	miR-29a	miR-29b	miR-29c
GLDC	-0.81	-0.70	-0.58

[a]Information involving the miR-29 family for 9 of the 900 total study participants was not available. miR, microRNA; GLDC, glycine dehydrogenase.

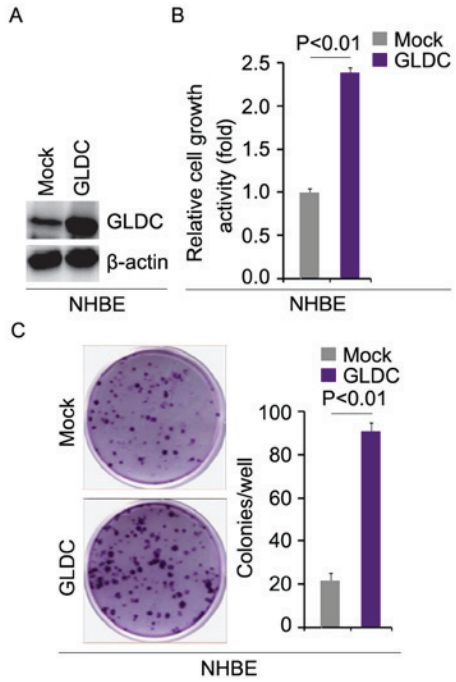

Figure 1. GLDC promotes tumorigenesis in NHBE. (A) Western blot analysis was performed to detect GLDC protein expression in NHBE cells transfected with GLDC expressing plasmids and pcDNA3.1 empty vectors (mock; n=3). (B) An MTT assay was conducted using NHBE cells transfected with GLDC expressing plasmids or pcDNA3.1 empty vectors (mock). P<0.01, as indicated. (C) Colony formation assay of NHBE cells transfected with GLDC expressing plasmids or pcDNA3.1 empty vectors (mock). Colonies ≥50 cells were counted. The left column presents representative micrographs and right column is the quantification of colonies. Data are presented as the mean ± standard deviation of 3 independent experiments. NHBE, human bronchial epithelial cells; GLDC, glycine dehydrogenase.

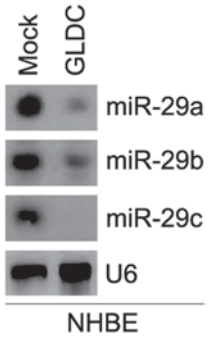

Figure 2. GLDC inhibits miR-29a/b/c expression in NHBE cells. Northern blot analysis for miR-29a/b/c in NHBE cells transfected with GLDC expressing plasmids or pcDNA3.1 empty vectors (mock; n=3). NHBE, human bronchial epithelial cells; GLDC, glycine dehydrogenase; miR, microRNA.

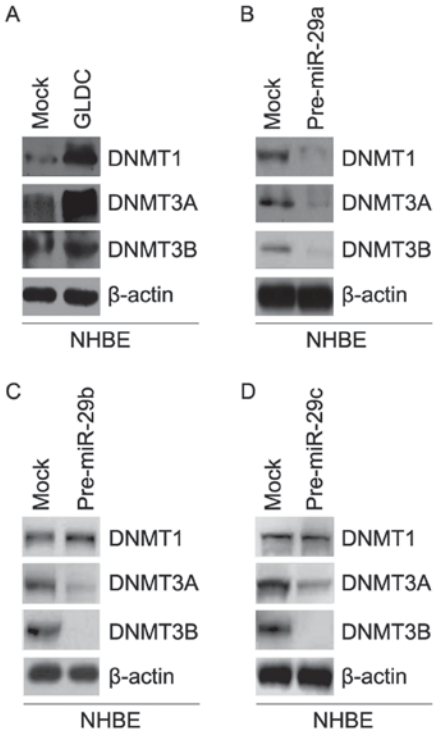

Figure 3. GLDC upregulates DNMTs expression, while the miR-29 family inhibits their expression in NHBE cells. Western blot analysis was performed to detect DNMTs protein expression in NHBE cells transfected with (A) GLDC expressing plasmids or pcDNA3.1 empty vectors (mock), (B) pre-miR-29a or control miR (mock), (C) pre-miR-29b or control miR (mock) or (D) pre-miR-29c or control miR (mock; n=3). NHBE, human bronchial epithelial cells; GLDC, glycine dehydrogenase; miR, microRNA; DNMT, DNA methyltransferase.

whether GLDC can affect DNMT1, DNMT3A and DNMT3B protein expression, western blot analysis was performed in NHBE cells transfected with GLDC expressing plasmids and empty vectors. The results of western blotting showed that DNMT1, DNMT3A and DNMT3B protein expression were upregulated by GLDC (Fig. 3A).

In addition, the results of western blotting were used to detect whether miR-29a/b/c can regulate DNMT1, DNMT3A and DNMT3B protein expression in NHBE cells. The results showed that miR-29a inhibited DNMT1, DNMT3A and DNMT3B protein expression in NHBE cells (Fig. 3B). However, miR-29b/c only downregulated DNMT3A and DNMT3B protein expression in NHBE cells (Fig. 3C and D).

GLDC is negatively correlated with miR-29a/b/c expression in the sera of participants. Having demonstrated that GLDC inhibited miR-29a/b/c expression in NHBE cells, the present study then used Spearman's correlation to analyze whether GLDC protein is negatively correlated with serum miR-29a/b/c expression. The results demonstrated that GLDC protein was negatively correlated with serum miR-29a/b/c expression in the serum of participants (Table VII).

Discussion

Experimental evidence has revealed that GLDC may be an oncogene in lung cancer (2); however, up to now, there has not been a perspective study that determined whether it can

promote the initiation of lung cancer. In this prospective study, an increased lung cancer risk was associated with higher serum GLDC concentrations. The results were in line with previous experimental evidence that demonstrated that exposure to GLDC can transform normal breast cells and primary NHBE cells to malignancy-like status (2). In order to reduce lung cancer mortality, prevention is one of the most effective strategies. Smoking is an important risk factor for lung cancer (31). Elucidating how carcinogens are produced by smoking and gaining a better understanding of this process will improve the scientific basis for the assessment of mechanisms associated with lung cancer development.

The present study showed that years of smoking were positively associated with the serum concentration of GLDC, which can increase lung cancer risk. The results implied that smoking may promote the initiation of lung cancer by upregulating serum GLDC concentration. Thus, developing an antagonist for serum GLDC may be helpful to prevent lung cancer in smokers.

In line with previous perspective and lab results (2), the present study revealed that GLDC promoted malignant transformation in NHBE cells. Increased proliferation and colony formation abilities are hallmarks of cancer (32). The results of the present study demonstrated that GLDC can promote proliferation and colony formation abilities in NHBE cells.

Aberrant methylation of TSG is an early event in the development of lung cancer (17,18). DNMTs control changes in methylation and 3 catalytically active DNMTs (DNMT1, DNMT3A and DNMT3B) have been identified (33). Recently, it has been reported that miRNA-29a/b/c can revert aberrant methylation in lung cancer by regulating DNMT3A and DNMT3B (7). In the present study, GLDC expression in serum, induced by smoking, was an upstream regulator of the miR-29 family. In addition, GLDC promoted DNMTs protein expression. Consistent with a previous report (7), the results of the present study showed that the miR-29 family may inhibit DNMTs protein expression in NHBE cells. Thus, the smoking/GLDC/miR-29 family/DNMTs signaling pathway may serve an important role in the early malignant transformation of normal lung cells.

Competing interests

The authors declare that they have no competing interests.

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