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Original Article

The effect of folic acid on epigenetic changes induced by MNNG in Kazakh esophageal epithelial cells and *DNMT1* high expression cells

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Abstract: In the present study, MNNG was used as a carcinogen, Kazakh esophageal epithelial cells and Kazakh esophageal epithelial cells with high expression of *DNMT1* were regarded as the subjects of research and folate was used as an intervention substance. High performance liquid chromatography was used to measure the overall methylation level of genomic DNA. Methylation specific PCR was used to detect the genes: *MTHFR*, *CBS*, *MGMT*, *P16*, *RASSF1A* and *FHIT*. The longer the MNNG acted, the lower the overall DNA methylation level and the higher the *DNMT1* enzymatic activity, and with the increase of folate concentration, the overall DNA methylation level rose and *DNMT1* enzymatic activity diminished gradually. Folate can reverse the methylation status of *MTHFR*, *RASSF1A* and *FHIT* genes in MNNG induced esophageal epithelial cells and *DNMT1* highly expressed cells, while having no effect on the methylation status of *CBS*, *MGMT* and *P16* genes. The overall DNA methylation level of *DNMT1* highly expressed cells was lower than that of Kazakh esophageal epithelial cells. In conclusion, Kazakh *DNMT1* overexpression cells are more susceptible to MNNG and low dose folic acid damage than esophageal epithelial cells. High concentration of folic acid can reduce the damage of MNNG on cells, reverse the changes of epigenetic indexes, and play an antagonizing role in the adverse reactions of cells. Therefore, folic acid supplementation could decrease esophageal cancer death among populations, which lays the theoretical foundation for studying the occurrence, development and prevention of esophageal cancer.

Keywords: DNA methyltransferase 1, folic acid, methylation, epigenetics, Kazakh

Introduction

Esophageal cancer is the eighth major malignant tumor type in the world. Its morbidity and mortality rates in China are among the highest. The residential area of the Kazakh ethnic group in Xinjiang province (hereinafter referred to as Kazakh) is one of the six high incidence areas of esophageal cancer in China. The incidence rate of esophageal cancer in Kazakhs is as high as 1,559/100,000, and its mortality rate (887/100,000) is much higher than other ethnic groups in the same area (223/100,000) [1]. Based on the current research, the abnormal methylation of DNA *CPG* islands is an early and frequent event in the process of tumor formation, and it is an important factor in tumor development. DNA methyltransferase 1 (*DNMT1*) is the most important enzyme that regulates DNA methylation, and it is involved in maintenance methylation. The activation and overexpression of *DNMT1* will lead to the silencing expression of tumor suppressor genes by promoting DNA hypermethylation so as to take part in the occurrence and development of tumors [2].

According to epidemiological studies, the risk factors for esophageal cancer include excessive intake of salted and smoked food, smoking, alcoholism, and lack of fresh fruits and vegetables in the daily diet [3, 4]. Excessive intake of pickled food can lead to the formation or accumulation of excessive harmful substances

including nitrosamines, benzopyrene and other polycyclic aromatic hydrocarbons. N-methyl-N'-nitro-n-nitroso-guanidine (MNNG) is a chemical mutagen and carcinogen that widely exists in our environment and it belongs to N-nitroso compounds. According to survey, the concentration of nitrate, nitrite and secondary amines in grains and drinking water has significantly increased in the high incidence areas of esophageal cancer in China, which is positively correlated with the prevalence of local esophageal cancer and severe hyperplasia of the esophageal epithelium. In this article, our study on Kazakh residential areas also obtained similar conclusions [5].

Folic acid is a water-soluble B vitamin mainly found in vegetables and fruits. It plays a key role in DNA synthesis and methylation through one-carbon metabolism. Mammals can't synthesize folate by themselves, so they have to get it from food. Folic acid in the form of 5'-methyl-THF is important in the transformation of proteins into S-adenomethionine (SAM). SAM directly provides methyl groups in DNA methylation. Without folic acid, the level of 5'-methyl-THF, the main circulating form of folic acid, will be low and SAM will soon be exhausted, which leads to the decrease of cytosine methylation in DNA. This study showed that severe folic acid deficiency causes hypomethylation of exons 6 and 7 of the *p*53 gene in mouse colonic mucosa cells, but this situation can partly be reversed by adding folic acid [6]. According to our study results also suggested that folic acid deficiency would reduce DNA methylation while adequate folic acid supply would reverse hypomethylation.

Therefore, in our study, MNNG was used as a carcinogen, Kazakh esophageal epithelial cells and Kazakh esophageal epithelial cells with high expression of *DNMT1* were regarded as the research subjects and folate was used as intervention substance. The *in-vitro* cell experiment studied how folic acid would change the indicators including the overall methylation of MNNG in Kazakh esophageal epithelial cells and *DNMT1* overexpressed epithelial cell DNA, as well as *DNMT1* enzyme activity and the methylation of folate metabolism-related genes (*MTFHR* and *CBS*), DNA damage and repair genes (*MGMT*) and tumor-related genes (*p*16, *RASSF1A* and *FHIT*); all of which lays a theoretical foundation for studying the occurrence, development and prevention of esophageal cancer.

Materials and methods

Reagents and instruments

Reagents: Kazakh esophageal epithelial cell line (self-made) [7], Kazakh esophageal epithelial *DNMT1* high expression cell line (self-made) [8], MNNG (TCI, Japan), epicm-2 culture medium (ScienCell, America), RPMI-1640 culture solution (Gbico, America), fetal bovine serum (Gibco, America), 0.25% trypsin, DMSO, MTT, folate powder (Sigma, America), SYBR Green PCR kit (Thermo, America), EPiQuikTM *DNMT1* Assay kit (EPigentek, America), antibody (Abcam).

Instruments: Fluorescence inverted microscope (Olympus, Japan), CO_2 incubator (SANYO, Japan), low temperature centrifuge (SONY, Japan), enzyme labeling instrument (Shanghai Xinzhen Instrument Co., Ltd.), nucleic acid electrophoresis instrument (Thermo, America).

Methods

Cell culture: Take out the cryopreservation tube containing Kazakhs' esophageal epithelial cells and esophageal epithelial *DNMT1* overexpressing cells and immediately put them into a container filled with 37°C water. After melting, centrifuge them (1500 r/min, 5 min), remove the supernatant, add 5 mL of 1 × epicm-2 complete culture medium (with folate content of 0.80 μg/M) to suspend the cells, and inoculate them in a 25 cm^2 culture bottle, until the cells grow to 80%-90%. Perform routine digestion and subculture them in 1:2.

Experimental grouping and intervention: In a 25 cm^2 culture flask, when the cells grew to 70%-80%, MNNG with final concentration of 1.5×10^{-5} mol/L and culture solution with folate concentration of 0.10 μg/mL, 1.00 μg/mL and 10.00 μg/mL were added respectively for one-hour intervention. The cells were washed by PBS three times. Then they were digested with 0.05% trypsin. The cells reproduced to a 1:2 ratio, during which we respectively used culture medium with folate concentration of 0.10 μg/mL, 1.00 μg/mL and 10.00 μg/mL according to the corresponding grouping for

Table 1. Methylation specific PCR primer sequence

Primer pair	upstream primer 5'→3'	downstream primer 5'→3'	T/°C	length/*bp*
MTHFR-P1	GAGGGGTTATGAGAAAAGATTTT	ACTCCTAATCTCAATCCCAAAA	59	406
MTHFR-M	GTGCGGGTTTTACGTTTATC	GAAAAAACCACGTAACCGTC	60	174
MTHFR-U	GGGTGTGGGTTTTATGTTTATT	AAAAAAACCACATAACCATCCC	60	176
CBS-P1	GTGTTAGTTTTTGTTAGTGGATATTT	ACTAAACCTAATCCCCCC	57	276
CBS-M	TTTTACGTGGTAGAGATCGC	AACCTACAACGAAAAACACG	60	148
CBS-U	TTTTATGTGGTAGAGATTGT	AACCTACAACAAAAAACACAAAC	60	148
MGMT-P1	GGTATTGGGAGTTAGGATTTTA	TTTTCCTATCACAAAAATAATCC	56	423
MGMT-M	TATAGGTTTTGGAGGTTGTTTTTAC	TAATAAAAATCCCGATCCTACTCG	58	147
MGMT-U	ATAGGTTTTGGAGGTTGTTTTTATG	AATAAAAATCCCAATCCTACTCAAA	58	145
P16-PCR1	TTAGATAGAAAGGTGGTATGTGG	CCAAACCTTACAAAAAAAAAAA	55	319
P16-M	ATTTTGAGTGAAATTTATTTATCGG	ATACAAACCCAAAACAAAACGAA	58	131
P16-U	ATTTTGAGTGAAATTTATTTATTGG	ATACAAACCCAAAACAAAACAAA	58	131
FHIT-P1	TTTAGAAAGATTTAGAGTGGGGA	AAACTACAATTCCCAAAAAACC	58	398
FHIT-M	AGAAATTTAGTTAGTGGGAAGTCGT	AAAAAAATTTAAAACATAAATCGCA	60	167
FHIT-U	AGAAATTTAGTTAGTGGGAAGTTGT	AAAAAAATTTAAAACATAAATCACA	60	167
RASSF1A-M	TTAGCGTTTAAAGTTAGCGAAGTAC	AAAATCGCACCACGTATACGTA	60	241
RASSF1A-U	TTAGTGTTTAAAGTTAGTGAAGTATGG	CACAAAAATCACACCACATATACATA	60	245

normal culture. This method was applied to every other generation. We collected cells and tested the relevant experimental indicators when the 9th, 21st and 27th generations were interfered with by poisoning. At the same time, we set the control group and observed the cell growth under an inverted fluorescence microscope.

Detection of the whole DNA methylation level: Use a DNA extraction kit to extract total DNA in cells, and use a nucleic acid analyzer to detect the absorbance ratio (A260/A280) at the wavelength of 260 nm and 280 nm to figure out the purity and concentration of DNA. Then treat the extracted genomic DNA with a DNA hydrolysis kit, and detect the methylation level of genomic DNA by HPLC. Meanwhile, draw a standard curve and formulate standard equation.

Detection of DNMT1 enzyme activity: Detect *DNMT1* enzyme activity according to an enzyme activity detection kit.

Detection of gene methylation: Detect the *MTHFR*, *CBS*, MGMT, *p16*, *RASSF1A* and *FHIT* gene methylation by methylation specific PCR (MSP).

DNA extraction: Use the DNA extraction kit to extract DNA, and strictly follow the operation steps of the kit, then dissolve the extracted DNA in 200 μL TB buffer, and store it at -20°C.

Genome methylation modification: Follow the operation steps of DNA methylation modification kit, and store it at -20°C.

PCR amplification and result determination: The modified DNA is used as the template for PCR, and the primer sequence is shown in **Table 1**. First, make PCR amplification: the first round of amplification system: DNA template 1 μL, 5 × KAPA 2G buffer A 5 μL, 5 × KAPA enhancer 15 μL, dNTP mix (10 mm) 0.5 μL. The upstream and downstream primers are both 1 μL, KAPA 2GRobot HotStart (5u/μL) is 0.1 μL, ddH_2O_2 is 11.4 μL, a total of 25 μL. Reaction conditions: pre-denaturation for 5 min at 94°C, denaturation for 30 s at 94°C, annealing for 30 s (see **Table 1** for annealing temperature of each gene), extension for 30 s at 72°C, with 35 cycles in total, repair extension for 7 min at 72°C. The second round of amplification system: DNA template 1 μL, 10 × MSP PCR buffer 2 μL, dNTP (2.5 mM) 2.5 μL. The upstream and downstream primers are both 1 μL, DNA polymerase (2.5U/μL) is 0.4 μL, ddH_2O_2 is 13 μL, a total of 20 μL. Reaction conditions: 95°C for 5 minutes, 94°C for 20 seconds, annealing for 30 seconds, 72°C for 20 seconds, with a total of 35 cycles, repair extension for 5 min at 72°C. The amplified products of 10 μL PCR are subject to 1% agarose gel electrophoresis. Results criteria: the amplified non-methylated bands are considered as unmethylated, while the am-

plified methylated and non-methylated bands are considered as partly methylated, and the amplified methylated bands are judged as completely methylated.

Data analysis

SPSS statistical analysis software was used to analyze the data. The measurement data was expressed in the form of mean ± SD ($\bar{x}$±s). Meanwhile we used the one-way ANOVA and trend test. SNK test was used to compare the two different groups, and the data was statistically significance with $P < 0.05$.

Results

Folate and DNA methylation level

In the Kazakhs' esophageal epithelial cell groups, after the long-term effect of MNNG and the intervention of folic acid in different concentrations, it was found that the overall DNA methylation level of each concentration group was different in the early, middle and late stages. The difference had statistical significance (F_{early} = 34.643, $P < 0.01$, F_{middle} = 277.121, $P < 0.01$, F_{late} = 67.751, $P < 0.01$), and the methylation level increased as the folic acid concentration grew (F_{early} = 45.510, $P < 0.01$; F_{medium} = 327.516, $P < 0.01$; F_{late} = 78.807, $P < 0.01$). The level of DNA methylation was significantly different in each time period among the control group, low, medium and high dose folate group groups ($F_{control\ group}$ = 2153.902, $P < 0.01$, $F_{low\ dose\ group}$ = 963.496, $P < 0.01$, $F_{medium\ dose\ group}$ = 493.935, $P < 0.01$, $F_{high\ dose\ group}$ = 56.966, $P < 0.01$). As the intervention time extended, the overall DNA methylation level of each group decreased ($F_{control\ group}$ = 3746.682, $P < 0.01$, $F_{low\ dose\ group}$ = 1674.249, $P < 0.01$, $F_{medium\ dose\ group}$ = 820.609, $P < 0.01$, $F_{high\ dose\ group}$ = 113.907, $P < 0.01$).

In the group with high expression *DNMT1* in the Kazakh esophageal epithelium, the overall DNA methylation level of each concentration group varied in the early, middle and late stages. The difference had statistical significance (F_{early} = 163.805, $P < 0.01$, F_{middle} = 1495.632, $P < 0.01$, F_{late} = 1640.957, $P < 0.01$). With the increase of folate concentration, the overall DNA methylation level grew (F_{early} = 184.966, $P < 0.01$, F_{middle} = 1254.430, $P < 0.01$, F_{late} = 2668.960, $P < 0.01$), the overall DNA methylation level of cells in each time period was different in the control group, low, medium and high dose folate groups, ($F_{control\ group}$ = 3587.688, $P < 0.01$, $f_{low\ dose\ group}$ = 3637.755, $P < 0.01$, $F_{medium\ dose\ group}$ = 52.768, $P < 0.01$, $F_{high\ dose\ group}$ = 76.579, $P < 0.01$). As the intervention time extended, the overall DNA methylation level of each group decreased. ($F_{control\ group}$ = 7029.611, $P < 0.01$, $F_{low\ dose\ group}$ = 7225.543, $P < 0.01$, $F_{medium\ dose\ group}$ = 103.911, $P < 0.01$, $F_{high\ dose\ group}$ = 149.823, $P < 0.01$). See **Table 2**.

After a long-term effect of a certain dose of MNNG and under the intervention of different concentrations of folic acid, it was found that in the late stage, the overall DNA methylation level of the esophageal epithelial cells were higher than that of the Kazakh esophageal epithelial cells with high expression of *DNMT1*. The difference had statistical significance (T_{early} = 0.696, P = 0.494, T_{middle} = 0.605, P = 0.551, T_{late} = 2.746, P = 0.012).

Folate and DNMT1 enzyme activity

With the long-term effect of MNNG and the intervention of different concentrations of folate, the *DNMT1* enzyme activity of each concentration group was different in the early, middle and late stages (F_{early} = 188.922, $P < 0.01$, F_{middle} = 709.777, $P < 0.01$, F_{late} = 484.207, P < 0.01). With the increase of folate concentration, the *DNMT1* enzyme activity decreased (F_{early} = 187.091, $P < 0.01$, F_{middle} = 595.047, $P < 0.01$, F_{late} = 224.427, $P < 0.01$), *DNMT1* enzyme activity level in each time period was different among the control cell group, compared to the low, medium and high dose folic acid group. The difference had statistical significance ($F_{low\ dose\ group}$ = 199.149, $P < 0.01$, $F_{middle\ dose\ group}$ = 80.477, $P < 0.01$, $F_{high\ dose\ group}$ = 414.273, $P < 0.01$). With the extension of intervention time, *DNMT1* enzyme activity of the cells in each concentration group was enhanced ($F_{low\ dose\ group}$ = 388.376, $P < 0.01$, $F_{medium\ dose\ group}$ = 154.556, $P < 0.01$, $F_{high\ dose\ group}$ = 828.409, $P < 0.01$). In different stages, the *DNMT1* enzyme activity of the cells in the medium and high dose folic acid groups was significantly different from that in the low dose group, and the *DNMT1* enzyme activity of the cells in the medium dose group was significantly different from that in the control group ($P < 0.01$). The difference had statistical significance.

Table 2. Comparison of the overall DNA methylation level of each intervention group (n = 3, $\bar{x}\pm s$, %)

Grouping		Esophageal epithelial cells of Kazakhs			variance analysis		High expression of *DNMT1* in esophageal epithelium of Kazakhs			variance analysis	
		early stage	middle	late	*F*	*P*	early stage	middle	late	*F*	*P*
Control cell group		3.755±0.104	1.222±0.002	0.930±0.013	2153.682	0.001	3.502±0.051	2.537±0.037	0.726±0.004	3587.688	0.001
MNNG+Folic acid low dose group		2.988±0.126	0.919±0.003	0.836±0.018	963.496	0.001	2.493±0.033	2.493±0.033	0.515±0.019	3637.755	0.001
MNNG+Folic acid medium dose group		4.124±0.143$^{\Delta}$	2.765±0.196$^{**,\Delta\Delta}$	1.677±0.013$^{**,\Delta\Delta}$	493.935	0.001	3.561±0.176$^{\Delta\Delta}$	3.215±0.050$^{**,\Delta\Delta}$	0.828±0.190$^{**,\Delta\Delta}$	52.768	0.001
MNNG+High dose folic acid group		4.980±1.004**	4.274±0.299**	2.841±0.456**	56.966	0.001	4.457±0.422**	4.182±0.076**	1.703±0.035**	76.579	0.001
variance analysis	*F*	34.643	277.121	67.751			163.805	1495.632	1640.957		
	P	0.001	0.001	0.001			0.001	0.001	0.001		
Trend test	*F*	45.510	327.516	78.807			184.966	1254.430	2668.960		
	P	0.001	0.001	0.001			0.001	0.001	0.001		

Note: campare with folic acid low dose group, $^{*}P < 0.05$, $^{**}P < 0.001$; compare with control cell group, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.001$.

In the group with high expression *DNMT1* in the esophageal epithelium of Kazakhs, *DNMT1* enzyme activity was different in the early, middle and late stage after the long-term effect of MNNG and under the intervention of different concentrations of folic acid. The difference had statistical significance (F_{early} = 1346.146, P < 0.01, F_{middle} = 455.866, P < 0.01, F_{late} = 2170.956, P < 0.01). With the increase of folate concentration, *DNMT1* enzyme activity decreased. The *DNMT1 enzyme* activity was different in the control group and the low, medium, high dose folic acid groups and the difference had statistical significance ($F_{low\ dose\ group}$ = 1034.793, P < 0.01, $F_{medium\ dose\ group}$ = 32.342, P < 0.01, $F_{high\ dose\ group}$ = 267.797, P < 0.01). As the intervention time extends, *DNMT1* enzyme activity of the cells in each concentration group shows an increasing trend ($F_{low\ dose\ group}$ = 2068.966, P < 0.01, $F_{medium\ dose\ group}$ = 643.282, P < 0.01, $F_{high\ dose\ group}$ = 534.398, P < 0.01). In different stages, the *DNMT1* enzyme activity of the cells in the medium and high dose folic acid groups was significantly different from that in the low dose group, and the *DNMT1* enzyme activity of the cells in the medium dose group was significantly different from that in the control group (P < 0.01). The difference had statistical significance. See **Table 2**.

With a long-term effect of MNNG and the intervention of different doses of folic acid, under the same conditions, there was no statistical difference in *DNMT1* enzyme activity between the two groups (T_{early} = 1.163, P = 0.257, T_{middle} = 1.338, P = 0.195, T_{late} = 1.255, P = 0.223).

Folate and DNA methylation status

MTHFR gene methylation assay: In the esophageal epithelial cells of Kazakhs, the specific gene fragment (176 *bp*) of *MTHFR* without methylation could be seen in the cells of each concentration group after the long-term effect of MNNG, along with the results from different concentrations of folic acid. However, in the late stages of the control cell group and the middle stage of the low-dose folic acid group, the methylated specific gene fragment (174 *bp*) of *MTHFR* gene, which is partly methylated, could be seen, indicating that with the long-term effect of MNNG and low-dose folic acid, the *MTHFR* gene is methylated. At the same time, with the increase of folic acid concentration, the *MTHFR* gene in cells remains unmethylated.

In Kazakh esophageal epithelial *DNMT1* overexpressing cells, with the long-term effect of MNNG and an intervention of different concentrations of folate, the unmethylated specific gene fragment (176 *bp*) and methylated specific gene fragment (174 *bp*) of *MTHFR* which is partly methylated, were found in the cells of control group, low dose group and medium dose folate group, as well as in the early stage of high dose group. However, only the 176 *bp* of the *MTHFR* unmethylated specific gene fragment was found in the middle and late stages of the high dose folic acid group, indicating that *MTHFR* gene could transform from partly methylated into unmethylated with the increase of folic acid concentration. See **Figure 1**.

Methylation determination of the RASSF1A gene: In Kazakh esophageal epithelial cells and *DNMT1* overexpressing cells, under the long-term effect of MNNG and intervention of different concentrations of folate, the unmethylated specific gene fragment (245 *bp*) and methylated specific gene fragment (241 *bp*) of *RASSF1A* gene, which is partly methylated, was found in the cells of control group, low and medium dose folate groups at different stages. However, only the unmethylated specific gene fragment (245 *bp*) of *RASSF1A* gene was found in the middle and late stages of the high dose folic acid group; indicating that with the increase of folic acid concentration, the *RASSF1A* gene in Kazakh esophageal epithelial cells and *DNMT1* high expression cells could change from being partly methylated to unmethylated. See **Figure 2**.

FHIT gene methylation assay: In the esophageal epithelial cells of the Kazakhs, the *FHIT* gene unmethylated specific gene fragment (167 *bp*) was found in the cells of each concentration group after the long-term effect of MNNG and the intervention of different concentrations of folic acid; indicating that the *FHIT* gene is not methylated in the cells of each group under the long-term effect of MNNG and after the intervention of different concentrations of folic acid.

In Kazakh esophageal epithelial *DNMT1* high expression cells, after long-term effect of MNNG and the intervention of different concentrations of folate, the unmethylated specific gene fragment (167 *bp*) and methylated specific gene fragment (167 *bp*), which is partly methylated, of the *FHIT* gene were found in the cells of control group, low and medium dose folate

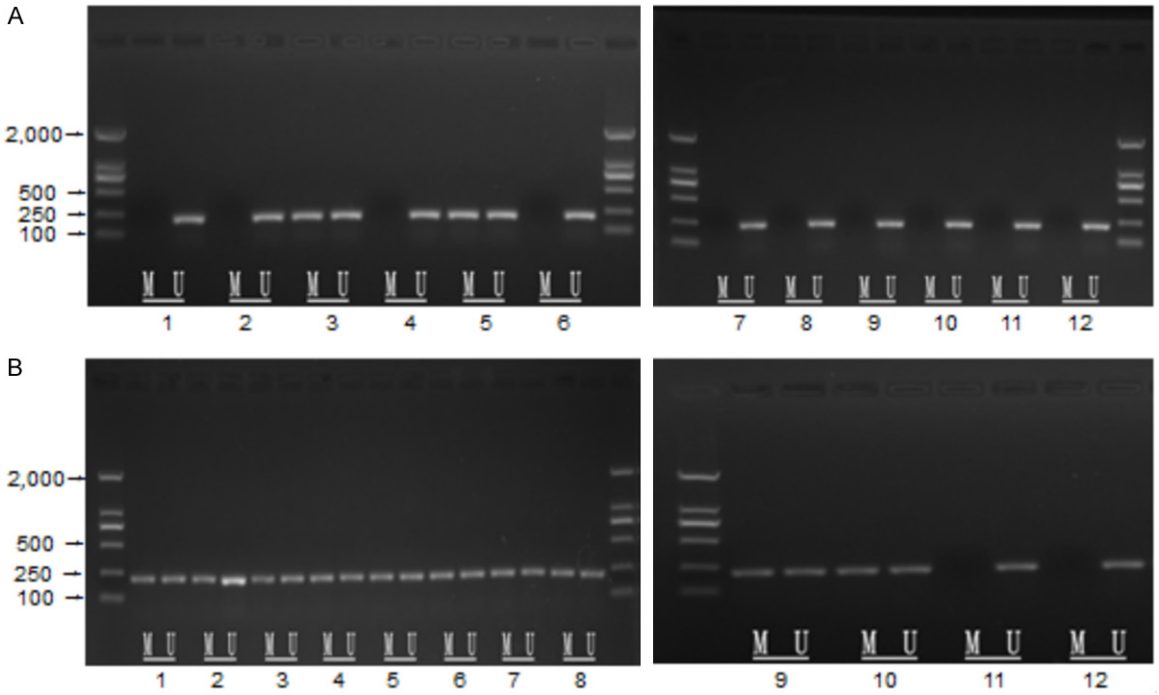

Figure 1. Electrophoretogram of *MTHFR* gene amplification products detected by MSP. A: Electrophoretogram of *MTHFR* gene amplification products in esophageal epithelial cells of Kazakhs. B: Electrophoretogram of *MTHFR* gene amplification products of *DNMT1* highly expressed cells in the esophageal epithelial cells of Kazakhs. Mark unit is bp, M: *MTHFR*-m specific primer amplification is 174 bp; U: *MTHFR*-u specific primer amplification band is 176 bp. 1, 2 and 3 respectively represents early, middle and late stage of the control cell group; 4, 5 and 6 respectively represents early, middle and late stage of the low dose folic acid group; 7, 8 and 9 respectively represents early, middle and late stage of the medium dose acid group; 10, 11 and 12 respectively represents early, middle and late stage of the high dose folic acid group.

groups, as well as in the early stage of high dose folate group. However, only the unmethylated specific gene fragment (167 *bp*) of the *FHIT* gene was found in the middle and late stages of the high dose group; indicating that with the increase of folic acid concentration, the *FHIT* gene in cells could change from a partly methylated state into an unmethylated state. See **Figure 3**.

Methylation determination of CBS, MGMT and p16 genes: The methylation of *CBS*, *MGMT* and *p16* genes did not change in the Kazakh esophageal epithelial cells and *DNMT1* high expression cells after long-term effect of MNNG and different concentrations of folate.

Discussion

With the development of molecular biology, epigenetics has been given more and more attention in the field of cancer prevention and treatment. DNA methylation, as one of the major methods of epigenetic change, is an important way to regulate human genetic modification and the only natural chemical modification of human DNA. It plays an important role in gene expression and regulation, cell proliferation and differentiation, development, gene imprinting, and is closely related to tumor occurrence and development. Because of the reversibility of epigenetic mechanisms, it is possible to prevent and control cancer through nutrients. The study of epigenetics, especially on folate, DNA methylation, signal transduction pathway regulation and tumors, has become research hotspot.

DNA methylation is essential for mammalian development; it can affect genome imprinting, *X*-chromosome inactivation, silencing of the genome and reverse transcription of the genome. DNA methylation mainly occurs at *CPG* diploid sites. In the human genome, about 80% of *CPG* dinucleotides are scattered, and a few of them are in local aggregation distribution, which is called a *CPG* island. Research shows that about 50% of *CPG* islands are located in

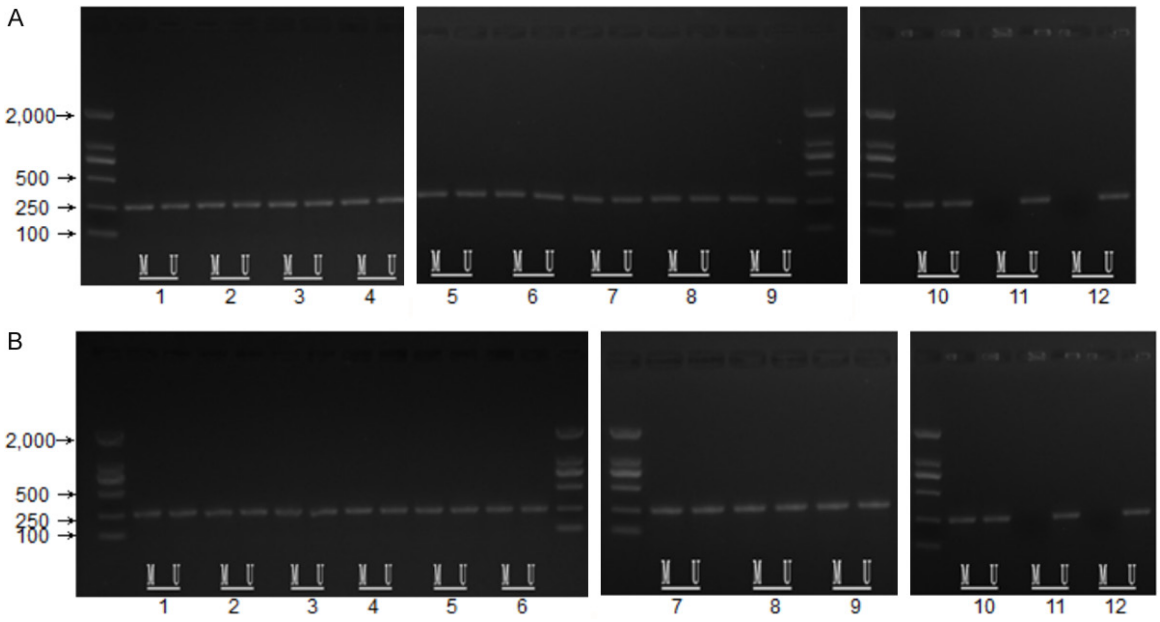

Figure 2. Electrophoretogram of *RASSF1A* gene amplification products detected by MSP. A: Electrophoretogram of *RASSF1A* gene amplification products in esophageal epithelial cells of Kazakhs. B: Electrophoretogram of *RASSF1A* gene amplification products of *DNMT1* highly expressed cells in the esophageal epithelial cells of Kazakhs. Mark unit is bp, M: *RASSF1A*-m specific primer amplification is 241 bp; U: *RASSF1A*-u specific primer amplification band is 245 bp. 1, 2 and 3 respectively represents early, middle and late stage of the control cell group, 4, 5 and 6 respectively represents the early, middle and late stage of the low dose folic acid group, 7, 8 and 9 respectively represents folic acid group early, middle and late in the medium dose group, 10, 11 and 12 respectively represents early, middle and late stage in the high dose group.

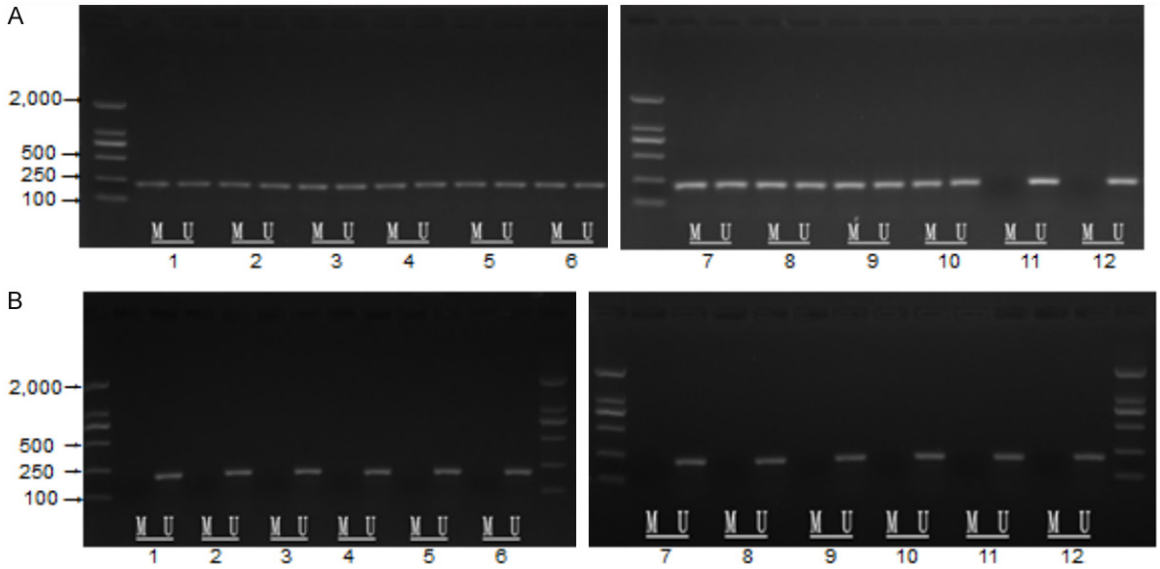

Figure 3. Electrophoretogram of *FHIT* gene amplification products detected by MSP. A: Electrophoretogram of *FHIT* gene amplification products in esophageal epithelial cells of Kazakhs. B: Electrophoretogram of *FHIT* gene amplification products of *DNMT1* highly expressed cells in the esophageal epithelial cells of Kazakhs. Mark unit is bp. M: *FHIT*-m specific primers amplification is 167 bp; U: *FHIT*-u specific primers amplification band is 167 bp. 1, 2 and 3 respectively represents early, middle and late stage of the control cell group; 4, 5 and 6 respectively represents early, middle and late stage of the low dose folic acid group; 7, 8 and 9 respectively represents early, middle and late stage of the medium dose group; 10, 11 and 12 respectively represents early, middle and late stage of the high dose group.

the promoter region of the gene, and half of the promoter regions in the human genome contain *CPG* islands. The *CPG* sites of *CPG* islands in a healthy human genome are usually unmethylated. When they are methylated, it will affect the regulation of gene transcription, resulting in gene expression silencing, and can then induce the occurrence of tumors.

In the process of tumor development, the methylation level of the whole genome is often reduced, and some specific oncogenes are in a low methylation state. The methylation of DNA silences tumor suppressor genes, which is one of the important reasons for tumor occurrence and development [9]. An experiment on the canceration of mouse esophagus induced by sodium nitrite n, n-dimethylbenzylamine showed that the DNA genome methylation modifications of mouse esophagus gradually decreased, indicating that a decrease of genome methylation modifications may be one of the causes of esophageal lesions induced by nitrosamines [10]. When the mice were fed with food free from folate, methionine, choline and vitamin B_{12} for one week, S-adenosylmethionine in hepatocytes decreased significantly, and DNA hypomethylation was found. When folate was supplemented, DNA methylation and the expression of the proto-oncogenes above gradually returned to a normal state [11]. It was found that the methylation level of lymphocyte genome was low in healthy individuals with insufficient folate intake, and when folate deficiency was corrected, the genome methylation returned to a normal state [12].

This *in-vitro* cell experiment applied high performance liquid chromatography to analyze Kazakh esophageal epithelial cells and Kazakh esophageal epithelial *DNMT1* overexpressing cells under the long-term exposure of the carcinogen MNNG, with a final concentration of 1.500×10^{-5} mol/L and the intervention of different concentrations of folic acid. It was found that with the extension of the intervention time, the overall methylation level of DNA in the genome of cells gradually decreased and there was a time-dependent relationship. The results showed that in the same time period, with the increase of folate concentration, the level of methylation in DNA of the genome gradually increased, and there was a concentration-dependent relationship. It can be seen from the results above that with the extension of the exposure time, the malignant transformation of cells induced by MNNG became more prominent, and the methylation level of DNA in the genome became lower. However, as the folic acid concentration increased, the methylation level of DNA in the genome increased to some extent, indicating that with sufficient folic acid, the overall methylation level of DNA in the genome of cells could be reversed or maintained. Our previous study [13] obtained similar conclusions. The results of MNNG and folate intervention showed that in the late stage, the methylation level of DNA in the genome in the Kazakh esophageal epithelial *DNMT1* overexpressing cells was lower than that in the Kazakh esophageal epithelial cells, indicating that the high expression of *DNMT1* enzyme somehow promoted the malignant transformation induced by MNNG, which shortened the time of malignant transformation.

At present, *DNMT*s mainly include *DNMT1*, *DNMT2*, *DNMT3a* and *DNMT3b*, among which *DNMT1* is the most important enzyme regulating DNA methylation, and it is mainly involved in methylation maintenance. *DNMT1* is a protein encoded by 1616 amino acid residues. It is located in 19*p*13.2-13.3 of the human chromosome and the cDNA has a total length of 5434 *bp*. It has been reported that *DNMT1* is highly expressed in various tumors, accompanied by an increase of enzyme activity [14]. Through research and comparison, the DNA methyltransferase activity in para-carcinoma tissues and lung cancer tissues is higher than that in normal tissues [15].

In the process of DNA methylation, folic acid is the main donor of methyl groups, while *DNMT*s play an important role in regulating DNA methylation and catalyzing the combination of methyl groups and cytosine rings. The biological relationship between folic acid and *DNMT*s suggested that the influence of folic acid on the occurrence and development of cervical cancer might be related to the change of *DNMT*s enzyme activity and function [16]. An animal experiment study found that folic acid could change the expression of *DNMT*s, thus affecting the growth of cancer cells [17]. This study found that after the long-term exposure of MNNG carcinogens and the intervention of different concentrations of folate, with the extension of intervention time, *DNMT1* enzyme activity of cells in each concentration group enhanced,

and there was a time-dependent relationship. Through comparing cells in each dose group within the same time period, it was found that *DNMT1* enzyme activity of cells in each intervention group decreased as the folate concentration increased, and there was a concentration-dependent relationship. The comparison between the two different folate dose groups suggested that the increase of folate dose might reduce the damage of MNNG to the cells by affecting the activity of *DNMT1* enzyme, so as to reduce the possibility of malignant transformation of esophageal epithelial cells.

The effect of folate on the methylation of folate metabolism related genes, Methylenetetrahydrofolate reductase (*MTHFR*) genes, was located at 1*p*36.3 of the human chromosome, and the whole coding region is 1980 *bp* long. It is the key enzyme in the process of folate metabolism. *MTHFR* catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, and the latter one is the main form of folate in human blood circulation, providing methyl donors for conversion of homocysteine into methionine, and methionine further transferred into S-adenosylmethionine (SAM). SAM is the main methyl donor of *in vivo* methylation reaction and plays an important role in DNA methylation. The functional defect of the *MTHFR* gene, especially when folic acid is inadequately supplied or utilized, will prevent some substrates from being methylated normally. Pike et al. [18] found that the *MTHFR* gene had methylation problems in diffuse low-differentiated lymphoma. Meanwhile, in these tumors, there was hypermethylation of *CPG* islands near the gene with low expression or undetectable expression. Therefore, these results suggested that hypermethylation of the *MTHFR* promoter region might down-regulate gene expression. This phenomenon might be related to the decrease or deletion of *MTHFR* gene mRNA expression. The plunge of the number of translated proteins caused the decrease of 5-methyltetrahydrofolate and the accumulation of homocysteine in the blood, impeding the synthesis of methionine, the methyl donor. This would eventually lead to abnormal methylation levels of DNA, the activation of oncogenes and the inhibition of tumor suppressor genes, and cell canceration. This study showed that under the long-term effect of MNNG and after the intervention of different concentrations of folic acid for different time durations, the *MTHFR* gene changed from unmethylated state to methylated state as the folic acid concentration decreased. This conclusion is similar to our previous study [13]. In the high expression cells of *DNMT1* in the esophageal epithelium of Kazakhs, the *MTHFR* gene changed from a methylated state to unmethylated state as the folic acid concentration increased. These results indicated that folic acid played an important role in maintaining the normal methylation of *MTHFR* gene.

The cystathionine beta synthase (*CBS*) gene is located in the 21st human chromosome, near the telomere, and consists of 23 exons, with a total length of about 30000 *bp*. *CBS* is involved in the metabolism of folic acid and homocysteine (*Hcy*). The abnormal activity of *CBS* can lead to the accumulation of *Hcy* in the human body and significant increase of plasma homocysteine concentration. Tu Xiaohuang et al. [19] found that the methylation level of the *CBS* gene in colorectal cancer patients was significantly higher than that in normal tissues ($P < 0.001$). Meanwhile, multivariate analysis showed that the methylation level of the *CBS* gene was an independent factor affecting the prognosis of colorectal cancer patients ($P < 0.05$), suggesting that the methylation level of *CBS* gene was closely related to the occurrence and development of tumors. In this study, we found that after long-term effect of MNNG and folate intervention, the *CBS* gene did not show methylated bands with the decrease of folate concentration and the extension of action time in Kazakh esophageal epithelial cells and *DNMT1* overexpressing cells. This might be caused by the limitation that MSP generally selects 1-2 *CPG* sites when detecting gene methylation. The *CPG* sites in the promoter region of the *CBS* gene selected in this study might not be sensitive to the effect of external factors.

The O^6-methylguanine-DNA methyltransferase (*MGMT*) gene is located at 10q26 of the human chromosome, with a total length of about 170 *KB*, containing 5 exons and 4 introns. The *MGMT* gene is a DNA repair gene, which can transfer the alkyl active gene binding to guanine O^6 to its 145 cysteine residue, so that guanine in the DNA chain can be repaired in time. In ESCC, the methylation frequency of the

MGMT gene is 27-72%, and its abnormal methylation is related to lymph node metastasis in ESCC patients [20]. Taioli et al. [21] used the *MSP* method and found that the methylation of the *MGMT* gene promoter was related to the overall survival rate of patients and tumor recurrence. It was believed that the methylation of the *MGMT* gene promoter can be used as a prognostic marker of primary oral and throat cancer. This study used MSP for detection and found the same results as *CBS* gene methylation, namely the *MGMT* gene maintained an unmethylated state with the decrease of folate concentration in esophageal epithelium and *DNMT1 high expression* cells of Kazakhs. The results showed that the concentration and action time of folic acid had little effect on the methylation of the *CPG* site in the promoter region of the *MGMT* gene selected in this study.

The P16 gene is a tumor suppressor gene that was found in 1992. It is located at 9p21 of the human chromosome, with a total length of 8.5 *KB*, consisting of 2 introns and 3 exons. The abnormal methylation of the *p16* gene was related to the occurrence and development of various tumors, such as gastric cancer, breast cancer, colorectal cancer, head and neck cancer, papillary thyroid cancer, etc [22]. In this study, we found the same results as *CBS* and *MGMT* gene methylation, that is, the *p16* gene remained unmethylated as the folate concentration decreased in Kazakh esophageal epithelium and *DNMT1 high expression* cells. The results showed that the concentration and action time of folic acid had little effect on the methylation of *CPG* in the promoter region of the *p16* gene selected in this study.

RAS association domain family 1A gene (*RASSF1A*), located at 3*p*21.3 of the human chromosome, is a new type of tumor suppressor gene. Methylation of the *RASSF1A* gene leads to gene transcription silencing, which is the most important inactivation mechanism. Byun et al. [23] found that *RASSF1A* was inactivated by about 60% based on 150 gastric specimens (including 15 cancer cell lines). A meta-analysis on the association between *RASSF1A* gene methylation and esophageal cancer showed that they were highly correlated (*OR*= 11.700, 95% *CI*: 6.59-20.9, $z = 8.36$, $P < 0.001$) [24], and *RASSF1A* gene methylation played an important role in esophageal cancer. This study found that in the Kazakh esophageal epithelial cells and *DNMT1* high expression cells, the *RASSF1A* gene in each concentration group was partly methylated after the long-term effect of MNNG, except in the middle and late stages of the high dose folic acid group, indicating that MNNG and low dose folic acid had certain damage to cells. Meanwhile, with the increase of folic acid concentration, the *RASSF1A* gene was found changed from a methylated state to an unmethylated state, indicating that folic acid could reverse the methylation state of genes to a certain extent.

Fragile histidine triad (*FHIT*) gene is a new candidate tumor suppressor gene that was found in 1996. The *FHIT* gene is located at 3p14.2 of the human chromosome. Its cDNA consists of 10 exons and it results in a protein with a relative molecular weight of 16.8 *kd* and is composed of 147 amino acids. Kuroki et al. [25] detected the *FHIT* gene in esophageal cancer tissues, and the abnormal methylation rate was 50%. Lee et al. [26] detected the methylation of the *FHIT* gene in 257 cases of early esophageal squamous carcinoma, among which 85 cases (33%) had abnormal methylation, suggesting that the abnormal methylation of the *FHIT* gene might be a biochemical indicator for early diagnosis of esophageal squamous carcinoma. This study showed that the *FHIT* gene in the Kazakh esophageal epithelial cells was methylated after the intervention of MNNG and folic acid. The *FHIT* gene in the Kazakh esophageal epithelial *DNMT1* overexpressing cells changed from a methylated state to an unmethylated state with the increase of folic acid concentration. These results indicated that folic acid played an important role in maintaining the normal methylation of the *FHIT* gene.

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All authors helped in provision of experiments, data analy-

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