

Non linear relationship between level of folic acid in serum and apoptosis in human gastric mucosa: a cross-sectional study

Aida Ghaffari¹, Beitollah Alipour², Abdolrasoul Safaiyan², Jabiz Modaresi¹,
Mohammad Hosein Somi¹, Alireza Ostadrahimi^{*3}

ABSTRACT

Background: Gastric cancer is the fourth common malignancy and the second prevalent cause of cancer death in the world. Genetic and environmental factors play an important role in the development of this cancer. Nutritional and epidemiological studies have indicated that folate status modulates the risk of developing cancer and apoptosis has been reported to play a decisive role in precancerous changes. The aim of this cross-sectional study was to investigate whether there is any relationship between serum folic acid levels and apoptosis, as an early indicator of gastric cancer changes, in human gastric mucosa.

Materials and Methods: This cross-sectional study was conducted on 62 patients out of 98 subjects with over 18 years old that referred to 2 hospitals in Tabriz, Iran to undergo an upper gastrointestinal endoscopy. We measured the levels of folic acid in serum and apoptosis was detected by TUNEL technique. We tested polynomial curve to find the best fit between them.

Results: The positive relation between serum folic acid and apoptosis found in the present study and our results indicate that the final and the best fit curve was:

apoptosis = $1.898 + 1.57 \times 10^{-10} \times (\text{Folic acid})^8$.

Conclusions: Our study indicates the relation between level of folic acid in serum and apoptosis in human gastric mucosa was not linear and it is suggested more interventional and control study.

Keywords: Gastric cancer, Apoptosis, Folic acid, Polynomial curve.

زمینه و هدف: سرطان معده چهارمین سرطان شایع و دومین علت مرگ ناشی از سرطان در جهان است. عوامل ژنتیکی و محیطی نقش مهمی در ایجاد این بیماری ایفا میکنند. مطالعات اپیدمیولوژیک و تغذیه ای نشان داده اند که وضعیت فولات تعدیل کننده سرطان و آپوپتوز به عنوان یک تغییر پیش از سرطان است. هدف از این مطالعه مقطعی بررسی ارتباط بین سطح اسید فولیک در سرم و آپوپتوز، بعنوان نشانگر اولیه تغییرات سرطانی، در مخاط معده انسان است.

مواد و روشها: این مطالعه مقطعی بر روی 62 مورد از 98 بیمار سرطانی بالای 18 سال که به دو بیمارستان تبریز برای انجام آندوسکوپی دستگاه گوارش فوقانی ارجاع شده بودند انجام پذیرفت. ما سطح فولات را در سرم و آپوپتوز نیز با استفاده از تکنیک TUNEL اندازه گرفتیم. ما نمودارهای پلی نومیال را برای یافتن مناسبترین نوع ارتباط بین این دو متغیر را آزمون کردیم.

یافته ها: ما ارتباط مثبتی بین سطح فولات در سرم و آپوپتوز یافتیم به گونه ای که مناسبترین منحنی بدینصورت ارائه گردید: $\text{apoptosis} = 1.898 + 1.57 \times 10^{-10} \times (\text{Folic acid})^8$

نتیجه گیری: مطالعه ما نشان داد که ارتباط بین سطح فولات در سرم و آپوپتوز در مخاط معده انسان خطی نیست.

واژه های کلیدی: اسید فولیک، سرطان معده، آپوپتوز، منحنی پلی نومیال

1. Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
2. Department of Vital Statistics and Epidemiology, Faculty of Health and Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran.
3. Nutrition Research Center, Department of Nutrition, Faculty of Health and Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran.

* Corresponding Author:

E-mail: ostadrahimi@tbzmed.ac.ir.

Introduction

Prevalence of gastric cancer is high all around the world. Although, the prevalence of this cancer in the last 70 years was decreased, it still is the fourth common malignancy in the world and the second prevalent cause of cancer death. Incidence of gastric cancer is particularly high in East Asia, Eastern Europe and parts of Central and South America.¹ According to a population-based cancer registry, Iran has the highest rate of stomach cancer among the Middle East countries.²

Sporadic gastric cancer is the result of genotypic changes due to an adverse environment i.e. diet & *Helicobacter Pylori*.^{3, 4} Dietary habits and nutrients intake play an important role in both prevention and causation of gastric cancer.⁵

Among the environmental and nutritional factors, a high intake of the vitamin folic acid or folate has received much attention as it is involved in DNA synthesis, repairment, and methylation and thus may play an important role in DNA homeostasis and control.⁶ Additionally, epidemiological studies have suggested that subjects with low folate intake or low folate status were at risk of various cancers, including gastric cancer.^{7, 8}

Folate is an essential cofactor in the de novo biosynthesis of purines and thymidylate, which are fundamental elements for DNA synthesis. It is also the key methyl donor for the methylation of DNA, RNA, other proteins, and phospholipids.^{9, 10} In particular, folate deficiency causes DNA hypo-methylation that may promote cancer formation/progression by activation of proto-oncogenes, reactivation of transposable elements, and loss of imprinting.^{11, 12} Studies on folic acid have been confined mainly to determination of serum folic acid concentrations and study on dietary intake of folic acid.¹³ Carcinogenesis is a complicated process that included a number of mechanisms such as programmed cell death or apoptosis.^{14, 15} Moreover, it has been shown that apoptosis plays a fundamental role in precancerous changes in the gastric mucosa.¹⁶

Because apoptosis is an early indicator of carcinogenesis in gastric mucosa¹⁷ we assessed the relationship between level of serum folic acid and apoptosis in gastric mucosa of patients undergoing upper gastrointestinal en-

doscopy to find the best relation between them.

Methods and Materials

This cross-sectional study was carried out in Tabriz Shahid Madani and Imam Reza hospitals during October to December 2008. This study was approved by the ethics committee of Tabriz University of Medical Sciences.

Participants

Patients over 18 years old, referred to 2 main hospitals in Tabriz to undergo an upper gastrointestinal endoscopy were asked to participate in this study. For interested participants, a check list was filled to understand which exclusion criteria were present. Among 109 subjects were interested, finally a total of 98 subjects had no exclusion criteria. Participants who had at least one of the exclusion criteria excluded from the study.

The following are the exclusion criteria

Alcohol consumption, smoking, any type of cancer, gastric surgery, certain cancer syndromes (hereditary nonpolyposis colorectal cancer, familial adenomatous polyposis, Peutz Jeghers syndrome), familial gastric cancer (defined according to the criteria proposed by International Gastric Cancer Linkage Consortium), gastric polyp (detected either in previous or present endoscopy), Menetrier's disease, Pernicious anemia and non-elective endoscopy.

Informed consent

Participants who had no exclusion criteria were informed about the goals and the procedure of the study and a written informed consent was taken from all the selected subjects. They were advised that the participant may quit at any time and without providing any reasons.

Upper Gastrointestinal Endoscopy

A skilled gastroenterologist who was completely familiar with the inclusion criteria of the study performed an upper GI endoscopy and when any suspicious lesion or tumor was detected during the endoscopy, the subject would be excluded from study. Otherwise, two biopsy samples from the antrum were taken by the physician

and one of the two sample was immediately put in formalin 20% and was sent to clinical pathology laboratory for examination by a skilled pathologist and the second one was kept in buffered formalin 10% and was taken to histology laboratory in Tabriz faculty of medicine for detecting apoptosis by TUNEL (terminal deoxynucleotidyl transferase nick –end labeling) technique.

When the endoscopy finished, patient was guide to another room and after relaxing a trained nutritionist would complete a demographic Questionnaire for him/her.

An anthropometric measurement was also performed and body mass index (BMI) was calculated as: $BMI = \text{weight (kg)}/\text{stature(m)}^2$.

After 12 hours of fasting, blood samples were gathered and centrifuged at 4°C and 500 g for 10 minutes which separated the serum. Then serum was frozen at -80°C until it was analyzed for folic acid. Levels of serum folic acid were measured by Electro chemiluminescence using the Elecsys of 2010 Roche analyzer.

Pathology and TUNEL technique

Infection of Helicobacter Pylori (HP) affects the rate of apoptosis in gastric mucosa. Approximately 2% of epithelial cells in the normal stomach are apoptotic. In gastritis induced by HP- infection, epithelial proliferation and apoptosis are moderately increased, with approximately 8% apoptotic epithelial cells.¹⁸

On the other hand, infection by HP is common in Iran and the rate of infection has been reported to be 69 – 89 % in different parts of Iran.¹⁹ We decided to examine apoptosis in patients with HP–induced chronic nonspecific gastritis in antrum.

After excluding the subjects who did not have one or more of the above mentioned requirements, finally 62 samples entered the final part of the study. **Figure 1** shows the flow chart of the participants of the study.

Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate end labeling (TUNEL) method.

In this phase, sections (4 thick) were cut from paraffin embedded blocks and mounted on microscope slides. The slides were incubated in for 36 hours before being de-paraffinized after those sections were de-paraffinized and rehydrated in a graded alcohol series, and incubated with proteinase K (Roche diagnostics, Germany) in for

30 minutes. TUNEL solution (Roche diagnostics, Germany) was prepared according to the instruction manual and the samples were incubated with this solution for 1 hour in . After washing, POD solution (Roche diagnostics, Germany) was added and the slides were incubated for another 30 minutes in .

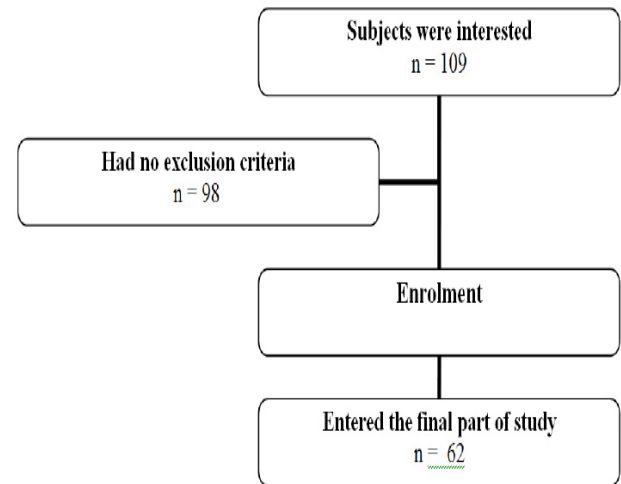


Figure 1- Study flow diagram of participants

Then di-amino banzidil (DAB) solution (Roche diagnostics, Germany) was added and after 15 minutes in room temperature, the slides were washed and counter stained with methylene blue. The prepared slides were examined under light microscopy (Nikon x 40). The number of apoptotic cells was counted in 10 high power fields (HPFs) for each slide and the mean number of apoptotic cells for each section was calculated.

Statistical analysis

For all continuous variables normality was tested by Q-Q test. All values are expressed as means ± SE at each time interval. Stepwise regression was exploited to access the relationship between apoptosis rate and serum folic acid.

The level of significance was set at P-value <0.05. Data was analyzed by SPSS version 16.00.

Results

62 subjects were finally enrolled in this study. Mean age was 43.60 ±1.67 years. The youngest par-

participant was 21 and the oldest was 65 years old with the same ethnic group (Iranian Turk), and 52.3% of the participants were female. Mean BMI was 25.31 ± 0.45 with a range 17.86-34.29. Mean folate concentration in serum was 11.18 ± 0.43 ng/ml with lowest 3.23 ng/ml and highest 20.00 ng/ml and mean for apoptosis in gastric mucosa was 2.15 ± 0.22 .

We tested polynomial curve (e.g. $Y = a + b_1x_1 + b_2x_2 + b_3x_3 + \dots + b_mx_m$ for $m = 1, 2, 3, \dots$) with different digress to find better fitting curve for showing complete relationship between folic acid and apoptosis. When we increase digress of polynomial curve from one to eight the large component digress power of polynomial remained in model with stepwise regression technique and removed other components with increase coefficient R in different models. After eight digress polynomial, coefficient R was constant and removed all of polynomial components except component with eight digress and removed all other components that our final result was apoptosis = $1.898 + 1.57 \times 10^{-10} \times (\text{Folic acid})^8$ (Table 1).

Discussion

Vitamins are essential for human life and deficiency of them cause various diseases, such as malignant neoplasia.^{20, 21} Recently, more studies show that deficiency or overdose of some vitamins increase susceptibility to cancer such as GI cancer.^{21, 22}

Folic acid plays an important and crucial role in DNA methylation and synthesis of DNA and RNA, and the synthesis of S-adenosylmethionine.²³ In addition, relationship between folic acid and apoptosis has not been elucidated.²⁴

We found positive relationship between serum folic acid and apoptosis. Our result was in agreement with many other epidemiological, animal and cell culture studies and somehow in contrast with some of them. The best fitting curve that shows complete relationship between folic acid and apoptosis was eight digress of polynomial curve.

Despite attention surrounding its relationship with carcinogenesis, the results of animal experiments were not consistent. Feeding Rats with low folic acid diet had decreased hepatic S-adenosylmethionine synthesis, resulting in DNA hypo-methylation.²⁵ Moreover, folic acid has a role in the development of cancer, such as colorectal cancer. There were abnormal breakages of chromosomes, incomplete contraction of bone marrow cells in patients deficient in folic acid.^{26, 27} In vitro cultured human lymphocytes with low amounts of folic acid leads to increasing DNA strand breakage and uracil disincorporation, in a time and concentration dependent manner.²⁸ That results to an increased risk of cancer in humans.²⁹ Da-Zhong Cao et al., showed that folic acid significantly increases the epithelial apoptosis and p53 expression in the gastric mucosa.³⁰ Mayne and colleagues reported that dietary intake of folate was inversely associated with the risk of

Table 1- Characteristics of different digress polynomial in fitting models for relationship between B9 and apoptosis

Digress of polynomial	Reminding component	R coefficient	constant	B coefficient	P-value
1	1	0.242	0.799	0.12	0.058
2	2	0.281	1.349	0.006	0.027
3	3	0.311	1.562	0.00033	0.014
4	4	0.334	1.681	1.84×10^{-5}	0.008
5	5	0.350	1.761	1.03×10^{-6}	0.005
6	6	0.359	1.818	5.61×10^{-8}	0.004
7	7	0.364	1.862	3.001×10^{-9}	0.004
8	8	0.364	1.898	1.57×10^{-10}	0.004
9	8	0.364	1.898	1.57×10^{-10}	0.004
10	8	0.364	1.898	1.57×10^{-10}	0.004

both oesophageal and gastric cancers (OR = 0.58, 95% CI = 0.39–0.85 for oesophageal squamous cell carcinoma and OR = 0.73, 95% CI = 0.55–0.97 for gastric cardia adenocarcinoma).³¹ It has been demonstrated that low folate status may increase the risk of malignancy with two principal mechanisms.³² First, deficiency of folate reduces intracellular S adenosylmethionine that can change cytosine methylation in DNA, resulting in inappropriate activation of proto-oncogenes and induction of malignant transformation. Second, folate is essential for DNA synthesis and repairment.

Bills ND and colleagues showed that folic acid supplementation had no protective effect on carcinogenesis and it even enhanced the development and progression of malignant tumor. In contrast, diminution of folic acid levels had an inhibitory effect on the development and growth of tumors.^{33, 34}

These conflicting results are probably due to effects of folic acid on tumors under different conditions, including different animal and tumor models used, differences in dosage of folic acid, timing of folic acid administration, variety of carcinogens, and methods of measuring folate.

In our study, we confirmed that the relation between serum folic acid and apoptosis was not linear and the best fitting curve that show complete relationship between folic acid and apoptosis was eight digress of polynomial curve. This result shows that the rate of apoptosis could be different by different concentration of serum folic acid. Presumably, the consequence of folate status and carcinogenesis depends on the balance between folate and the carcinogens. Different folate concentration affects apoptosis in different cells with different qualifications. However, increased intensity of the carcinogen may also lead to carcinogenesis even if folic acid levels in blood and tissue are within the normal range and in some conditions; high dosage of folic acid induces different cancer.

Results of this study may provide us with some additional clues about the mechanisms through which folic acid may alter the process of gastric carcinogenesis. Considering the high prevalence of this cancer in some parts of the world including Iran, these findings will help planning and supporting dietary habits (fruits and vegetables) and dietary supplementary of folic acid (the best dosage of folic acid). These actions will result in reducing the prevalence of this malignancy which is usually detected

in its final stages.

Advantage of the present study was its statistical method. Our study has several limitations. Because both cell proliferation and cell death influence the process of carcinogenesis assessing cell proliferation in addition to apoptosis rate could increase validity of our study. Furthermore in spite of the fact that TUNEL is a very well known method for detecting apoptosis, it suffers from some shortcomings. TUNEL will stain necrotic cells as well as apoptotic cells. On the other hand apoptosis can be induced, although rarely, without extensive deoxy-nucleotidyl transferase (DNA) degradation which is the phenomena detected by TUNEL. Thus, apoptosis detection exploiting TUNEL has false positives and false negatives. Moreover the length of time that tissue is left before fixation can also affect the results of TUNEL. So if a complementary method was added for detection of apoptosis, the results would have been more reliable. Another limitation is that many factors such as other vitamins and minerals can effects apoptosis. Because of the limitations of measuring serum folate, it is more reliable if we would consider RBC folate levels or concentration of folic acid as measuring tests. Larger interventional and control study with different micronutrient and different dosage are needed to characterize this relation.

Conclusions

Our study has shown that the relation between level of folic acid in serum and apoptosis in human gastric mucosa was not linear. As apoptosis is one of the effective components in the process of gastric cancer, it is worth designing more elaborate prospective and interventional studies to examine these findings more specifically. Studies performed under more controlled conditions such as cell culture studies, measuring level of folic acid in epithelial cells, rate of gastric cell proliferation and bester method for detecting apoptosis will also be very helpful in determining the effect of certain nutrients on apoptosis.

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Authors' Contributions

AG, JM and AO designed the study, JM and AG collected the data, AG and JM designed the experiments. AS and AG analyzed the data and AG wrote the manuscript. The authors would like to thank Mohammad Hosein Somi, Leila Roshangar for helping our in this study.

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