

Original Article

Correlation analysis of riboflavin, RFT2 and Helicobacter pylori in gastric carcinoma

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Abstract: Objective: To investigate the relationship between tissue riboflavin level and riboflavin transporter 2 (RFT2) protein expression, and the relationship between Helicobacter pylori (H.pylori) infection and the plasma riboflavin level in gastric carcinoma (GC). Methods: Enzyme-linked immunosorbent assay (ELISA) was used to detect tissue riboflavin level in patients with GC. Western blotting was applied to analyze the expression of RFT2 protein in 60 tissue samples from gastric carcinoma together with their normal tissues. The Warthin-starry method, rapid urease test and ¹⁴C-UBT were administered to detect the infection of H.pylori. High performance liquid chromatography (H.PYLORILC) was performed to detect plasma riboflavin level in the GC. Results: A significant decrease in the tissue riboflavin level was detected in GC samples compared to those in the normal mucous membrane (17.02 ± 3.91 vs. 21.0 ± 4.73 ; $P = 0.043$), and a significant decrease in the RFT2 protein was found in GC samples compared to those in the normal mucous membrane (0.92 ± 0.39 vs. 1.23 ± 0.51 ; $P = 0.042$). A positive correlation of tissue riboflavin level with defective expression of RFT2 protein was observed in GC patients ($\chi^2 = 1.969$; $P = 0.039$). Plasma riboflavin level in gastric cancer without H.pylori infection group ($1.6674 \text{ ng/mL} \pm 0.37009 \text{ ng/mL}$) was higher than H.pylori infection group ($1.2207 \text{ ng/mL} \pm 0.17727 \text{ ng/mL}$, $P = 0.043$). Conclusion: The results indicate that RFT2 plays an important role in gastric carcinogenesis by modulating riboflavin absorption. H.pylori infection affects plasma riboflavin level and the prognosis of patients with gastric cancer.

Keywords: Gastric carcinoma (GC), helicobacter pylori, RFT2, riboflavin

Introduction

Gastric cancer is one of the most common malignancies in China, and the third leading cause of cancer-related death worldwide. Xinjiang Uygur Autonomous Region is the area inhabited by various minority groups, with its unique geographical and cultural environment and life style. The incidence of gastric cancer is much higher in this region than any other places in China. The incidence and mortality rates of gastric cancer in China in 2009 were 36.21 and 25.88 per 100 000 people, respectively [1]. The incidence of gastric cancer is related to infection, diet, environment, nutrition, and genetics [2].

Riboflavin (vitamin B2) is an essential vitamin that is required for normal cellular functions, including growth and development in all aero-

bic forms of life. Riboflavin is phosphorylated intracellularly to FMN, and then further metabolized to FAD [3]. Riboflavin is known as cofactors for enzymes involved in oxidation-reduction reactions, and has a role in the oxidative folding of proteins in the endoplasmic reticulum [4, 5]. It has been reported that deficiency of riboflavin plays a prominent role in increased vulnerability of cells to cancer as well as progression of various cancers [6]. In addition, Riboflavin has also involved in the enhancement of antitumor activity of many anticancer drugs, as well as in the activation of the immune system to kill tumor cells [7-9]. Riboflavin transporter 2 (RFT2/SLC52A3/C20orf54) is a human riboflavin transporter that has an important role in the intestinal absorption of riboflavin [10]. It has been reported that riboflavin deficiency is associated with an increased risk of esophageal

squamous cell carcinoma (ESCC) and gastric cardiac adenocarcinoma (GCA), and riboflavin supplementation reduces the risk of ESCC and GCA [11-13]. Therefore, RFT2 may have an important role in modulating riboflavin absorption. Our previous study showed that defective expression of RFT2 is associated with the development of gastric cancer, and there is positive correlation between defective expression of RFT2 and plasma riboflavin level in gastric cancer patients [14].

Therefore, we hypothesized that human RFT2 is likely to have an important role in gastric carcinogenesis that involves modulating riboflavin absorption. To test this hypothesis, we investigated the tissue riboflavin status of gastric cancer and riboflavin transporter (RFT2) protein status in matched tissues, and further analyzed the relationship between RFT2 protein and riboflavin level in the GC tissues.

Helicobacter pylori (H.pylori) infection of the gastric mucosa is associated with gastric cancer. This has been confirmed by animal experiments, a large number of clinical observation and clinical intervention [15]. Nitrosamines have been suspected in the etiology of gastric cancer in the high incidence area of China. However, deficiencies in riboflavin, vitamins A and C and other micronutrients may also be involved.

Recent research [16, 17] has showed that the aetiology of GAC and esophageal cancer are related to lack of nutrition, low intake of fruit and vegetables. Wang et al. [18] reported that C20orf54 is a high susceptibility gene in GAC and esophageal cancer. C20orf54 gene is located in chromosome 20p13, consisting 5 exons, encoding riboflavin transporter protein. Its main function is to transport riboflavin from extracellular to intracellular [19]. Fujimura et al. [20] demonstrated that RFT2 is involved in the epithelial uptake of riboflavin in the small intestine. RFT2 is responsible for the transport of riboflavin, and riboflavin deficiency has been reported as a risk factor for gastric cancer. If C20orf54 gene is mutated, riboflavin cannot effectively enter the cells.

We also investigated the correlation of H.pylori infection and plasma riboflavin level in patients with gastric cancer.

Materials and methods

Clinical samples

A total of 60 frozen biopsy tissue specimen, consisting of 30 cases GC and 30 cases matched normal mucous epithelia (5 cm away from the tumor), were collected within 30 min after resection and kept at -80°C until tissue riboflavin levels by ELISA and RFT2 protein levels by western blots were tested. All GC patients were referred between May 2010 and Jun 2012 in their initial visits to the First Affiliated Hospital of the Xinjiang Medical University. Written informed consent was obtained from all patients participating in this study, and the study was approved by the ethics committee of the first affiliated hospital of Xinjiang medical university. Among patients with GC, 18 cases were tumor stage \geq stage III b, and 12 were \leq III a. 8 cases were pathologically characterized as well-differentiated, 11 moderately differentiated and 11 poorly differentiated tumors. Lymph node metastasis was documented in 19 cases. The mean age of GC was 55.7 years with extreme ages of 37 and 71.

A total of 60 cases with gastric cancer that underwent surgery at the Department of General Surgery of the first affiliate Hospital of Xinjiang Medical University from January 2010 to February 2011 were enrolled in the study. The H.pylori infection group: 32 cases; with the mean age of 61 years (range: 25~83 years); histological types of 18 cases of low differentiated adenocarcinoma, 12 cases of adenocarcinoma, 2 cases of high differentiation adenocarcinoma. No H.pylori infection group: 28 cases; with a mean age of 52 years (range: 36~75 years); histological types of 12 cases of low differentiated adenocarcinoma, 10 cases of adenocarcinoma, 6 cases of high differentiation adenocarcinoma. Age, gender, and tumor histology of these two groups showed no statistical significance. Peripheral blood samples were collected from 60 cases of gastric cancer patients (5 ml per person), put into EDTA vacutainer tubes and blend for 3~5 minutes. Then, the remaining samples were centrifuged 10 min (3000 r/min) and collected plasma was stored at -80°C.

All of the patients were enrolled with written informed consent, and the study was approved

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by the Ethical Committee of the Xinjiang Medical University. Patients had not received preoperative chemotherapy and/or radiotherapy. All patients were confirmed by gastroscopy, histopathological diagnosis and surgery.

Tissue riboflavin level

Tissue was analyzed for its concentration of riboflavin by ELISA. ELISA is a convenient method for measurement of riboflavin in tissue. In accordance with ELISA kit instructions, it was used to detect the riboflavin levels in fresh tissue from gastric carcinoma patients and matched normal mucous epithelia (5 cm away from the tumor). Fresh tissue (100 mg) from the same patient with gastric cancer and normal mucous epithelia 5 cm away from the tumor (control group) were weighed on an electronic balance, added to 1 ml of 10 mM PBS solution, then homogenized. The homogenate was centrifuged 20 min at 2000 rpm, and 200 μ l of supernatant was collected for analysis. The optical density (OD) of the supernatant was read within 15 min at 450 nm. A standard curve was used to determine the concentration of the sample.

Protein extraction and Western blotting analysis

Total protein from the same patient with fresh tissue of gastric cancer and gastric mucosa 5 cm away from the tumor tissue were extracted with radio immunoprecipitation assay (RIPA) lysis buffer (Biotek, Beijing, China) containing protease inhibitor. The proteins were separated by 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were incubated in blocking buffer (1 h with 5% skimmed milk in PBST) at room temperature with gently shaking. Then, the sample was incubated overnight at 4°C with primary antibody for anti-RFT2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with PBST 10 min \times 3 times, the membranes were incubated with horseradish peroxidase conjugated IgG at room temperature for 2 h. The blot was visualized using DAB kit (Zhongshan Jinqiao, Beijing, China). Western blotting band was quantified using Quantity One software by measuring the band intensity for each group and normalizing to β -tubulin (Sigma) as internal control (Invitrogen). The final

results were expressed as fold changes by normalizing data to the control values.

Detection of H.pylori infection

Without antibiotics, antisecretory drugs in 2 weeks, gastroscopy: H.pylori rapid urease test, Warthin2starry histological staining, and 14C-urea breath test (14C-UBT). Any 2 or more positive cases for H.pylori infection, 3 negative cases for no H.pylori infection, and only 1 positive cases were removed.

Detection of plasma riboflavin

Blood plasma was analyzed for its concentration of riboflavin by high-performance liquid chromatography (H.PYLORILC). The H.PYLORILC system used was a Waters 2695 liquid chromatograph and Waters 2475 fluorescence detector with the autosampler set at 28°C and configured for a 96-well microtiter plate (Sirocco™, Waters, 89013B). Water was generated using a Milli-Q water system (MILLIQ, serial0002). All chemicals were of analytical grade. For quality control, we used three Clin Chek serum controls, reconstituted and stored at -80°C. Aliquots of aqueous (0.3860 g/L C₂H₇NO₄) flavin stock solutions (5 mmol/L) were stored at -20°C in the dark. An excitation wavelength of 450 nm was used and riboflavin was detected at an emission wavelength of 520 nm. Chromatographic column: Symmetry-shield™ RP-C18, column (4.6 μ m \times 25 cm, 5 μ m); mobile phase: methanol (5 mmol/L) - ammonium acetate (volume ratio 35:65, pH 5.75), the flow rate was 0.60 ml·min⁻¹, the peak area was measured and used for quantification.

Statistical analysis

All statistical analyses were performed with SPSS Version 17 software. *P* values were two-sided, and the significance level was *P* < 0.05. The independent samples t-test was used to compare the quantitative variables. The χ^2 test was used to compare qualitative variables. Logistic regression analysis was used to compare the relationship among H.pylori infection, the plasma riboflavin level in gastric cancer. Results were presented as mean \pm SD. Variance analysis were used to analyze the association between two continuous variables.

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Table 1. RFT2 protein expression and riboflavin level in GC tissues

	Riboflavin level in tissues (means \pm SD $\mu\text{g/L}$)	<i>P</i>	Protein expression of RFT2	<i>P</i>
GC	17.02 \pm 3.91	0.039	0.92 \pm 0.39	0.042
Normal	21.01 \pm 4.73		1.23 \pm 0.51	

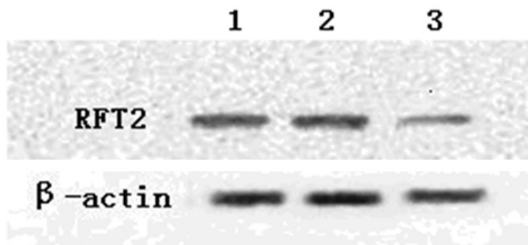


Figure 1. Expression of riboflavin transporter2 in gastric cancer tissue and normal tissue.

Results

Riboflavin levels are decreased in GC tissues

The average riboflavin level measured by ELISA in GC tissues was 17.02 \pm 3.91 $\mu\text{g/L}$, and the average level in tissues of matched normal gastric epithelial tissue was 21.01 \pm 4.73 $\mu\text{g/L}$. A decreased tissue riboflavin level was found in GC compared with normal gastric epithelial tissue (**Table 1**).

RFT2 protein expression is decreased in GC tissues

Based on Western blot analysis, expression of RFT2 protein was found to be lower in GC (0.92 \pm 0.39) compared to matched normal tissues (1.23 \pm 0.51) (**Figure 1**). The detailed results of RFT2 protein were summarized in **Table 1**. Statistical analysis demonstrated that expression of RFT2 in GC was significantly lower than that in normal tissues at protein level (**Table 1**).

Riboflavin level is correlated to RFT2 protein expression in GC tissues

RFT2 concentrations were determined in samples obtained from gastric cancer patients who were not taking vitamin supplements. The RFT2 protein expression level was 0.92 \pm 0.39 in patients, and the average tissue concentration of riboflavin was 17.02 \pm 3.91 $\mu\text{g/L}$ in GC patients. We have analyzed the relationship

between the tissue riboflavin level and expression of the RFT2 protein of GC. A positive association was found between changes in the tissue riboflavin level and changes in RFT2 protein expression in GC ($\chi^2 = 1.969$; $P = 0.039$).

Plasma riboflavin level is decreased in H.pylori infection

Plasma riboflavin level measured by H. PYLORILC of non-H.pylori infection group (1.6674 ng/mL \pm 0.37009 ng/mL) was higher than that of H.pylori infection group (1.2207 ng/mL \pm 0.17727 ng/mL); the difference in level of plasma riboflavin was statistically significant ($P = 0.043$, **Table 2**). We also found that plasma riboflavin levels are decreased in positive Serosal Invasion (vs. negative, $P = 0.013$) and lymph node metastasis (vs. negative, $P = 0.046$) samples (**Table 2**).

Discussion

The etiology of gastric cancer is particularly complex, influenced by diet, environment, genetics, immunization and other factors. In the present study, we investigated the association between tissue levels of riboflavin and the RFT2 protein expression status in patients with gastric adenocarcinoma. The results showed a tendency for a positive association between riboflavin levels and RFT2 expression status, which indicated that RFT2 may play an important role in gastric carcinogenesis and involve modulating riboflavin absorption.

Previous epidemiological studies have reported [21, 22] that riboflavin deficiency is linked to an increased risk of cancer because riboflavin is involved in essential oxidation-reduction reactions and its deficiency leads to skin and mucosal disorders. Furthermore, Riboflavin is essential for synthesis of FAD and FMN, which acts as a cofactor for several biological processes in energy metabolism. There are some studies showing that riboflavin is a potential cancer preventive agent because of its role in one carbon metabolism. Singh *et al.* reported that a reduced riboflavin intake can lead to changes in the methyl supply, followed by alterations in DNA methylation [23], and cancer cell lines showing that riboflavin can activate extrinsic apoptosis pathways at low concentration.

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Table 2. Plasma riboflavin level in serosal invasion, lymph node metastasis and helicobacter pylori infection

Characteristics	n	Riboflavin level (ng/mL)	P value
Serosal Invasion (Positive)	24	0.7331±0.11191	0.013
Serosal Invasion (Negative)	36	1.8706±0.27881	
N metastasis (Positive)	36	0.7950±0.12621	0.046
N metastasis (Negative)	24	2.3811±0.28812	
H.P infection (Positive)	32	1.2207±0.17727	0.043
H.P infection (Negative)	28	1.6674±0.37009	

Additional cell death mechanisms like intrinsic apoptotic pathways and protease pathways are also triggered at higher concentrations of riboflavin, leading to further inhibition in their proliferation [24].

Studies have reported that RFT2 is a human riboflavin transporter and a transmembrane protein, which may function biologically as a transporter of riboflavin in the small intestine [12], and riboflavin deficiency has been associated with an increased risk of esophageal squamous cell carcinoma (ESCC) and gastric cardia adenocarcinoma (GCA). Riboflavin supplementation has been reported to reduce the risk of ESCC and GCA [13], and RFT2 may have an important role in modulating riboflavin absorption. RFT2 is not only expressed in small intestine but also in other organ and tissue, just like jejunum ileum and testis, as well as in lung, kidney, stomach and colon [11]. Therefore, RFT2 not only plays important roles in intestinal riboflavin absorption but also effects riboflavin tissue distribution. Thus, RFT2 expressed in gastric tissue could play a key role in riboflavin handling.

It is proved that gastric cancer is closely related to helicobacter pylori infection. However, the carcinogenic mechanism is unclear. Many studies have confirmed that incidence of gastric cancer with the H.pylori infection is significantly higher than those without infection. The apoptosis and proliferation of antral mucosal cell increase in patients with H.pylori infected gastric cancer, the degree of increasing is closely correlated to that of inflammation. H.pylori may promote abnormal expression of certain genes, leading to gastric mucosa epithelial cell proliferation and apoptosis imbalance, causing abnormal gastric mucosa, eventually resulting in gastric cancer [25, 26]. In the present study, H.pylori infection can cause the oncogene acti-

vation and tumor-suppressor gene inactivation which contributes to the occurrence of gastric cancer.

This study showed that plasma riboflavin levels in gastric cancer with H.pylori infection group are lower than without H.pylori infection group, which proved that H.pylori infection can influence riboflavin absorption, accelerate the deterioration of gastric cancer, and shorten the lifetime.

It has been reported that the lifespan of gastric cancer with H.pylori positive is shorter than H.pylori negative [27]. Effective prevention and control of H.pylori infection, and increase riboflavin intake, might be of significance in the prevention, treatment and improvement the prognosis of gastric cancer.

Riboflavin, also known as vitamin B2, is two kinds of flavin coenzyme, and also is the important part of flavin mononucleotide and flavin adenine dinucleotide. These two kinds of coenzymes are combined with various proteins to form a flavoprotein, and participated in the biological oxidation reaction of organism and energy metabolism. Riboflavin deficiency can cause metabolism disorder and epithelial injury. Wynder observed pathological changes in the process of riboflavin deficiency in mouse epithelial tissue, such as gastric mucosa epithelial atrophy, excessive diversification and excessive hyperplasia, and these pathological changes are related to the incidence of esophageal cancer and gastric cancer. Our study suggested that plasma riboflavin level of gastric cancer without serosal invasion and lymph node metastasis is higher than serosal invasion and lymph node metastasis group. Consistent with literature, lack of vitamin B2 has close relationship with a high incidence of gastric cancer and gastric cancer prognosis. As far as current information is concerned, increase the dietary intake of riboflavin in the areas of high incidence of cancer especially esophageal and gastric cancer may be beneficial to the prevention of cancer. So far, domestic studies have been done on the measurement of vitamin A, vitamin B2, vitamin C in the blood of normal people in high incidence of esophageal cancer areas, but no studies have been reported on the relationship between gastric cancer helicobacter pylori infection and blood riboflavin level. This study provides a valuable objective data

for etiology and prevention measures of gastric cancer.

In recent years, it has been found that H.pylori also causes the deficiency of iron, copper, vitamin and micronutrient, which will lead to iron deficiency anemia, pernicious anemia, and even some tumors [28]. Based on the literature, the riboflavin absorption barrier mechanism caused by H.pylori infection is unclear, but may be related to the damage of gastric epithelium after H.pylori infection, thus affecting the riboflavin absorption. Low plasma riboflavin level cannot guarantee the normal epithelium function, which otherwise causes H.pylori infection easily.

In conclusion, there is lack of strong evidence about a protective effect of riboflavin against gastric cancer, but this may be partly due to not considering RFT2 as a key risk factor. In the present study, we provided an evidence for insufficient riboflavin was associated with an increased risk of gastric cancer. Plasma level of riboflavin in H.pylori infection patients with gastric cancer is lower than that of non-H.pylori infection. We believe that the present of the relationships among helicobacter pylori infection, gastric cancer invasion, and metastasis and plasma riboflavin may provide new theories and methods for the prevention and treatment of gastric cancer.

Disclosure of conflict of interest

None.

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