Received: 2015.08.14
Accepted: 2015.10.29
Published: 2016.05.02

MicroRNA-421 Gene Polymorphism in Gastric Carcinoma

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCD **Xin Jin**
DEFG **Nong Yu**

Department of Clinical Laboratory, Affiliated Hospital, Academy of Military Medical Sciences, Beijing, P.R. China

Corresponding Author: Nong Yu, e-mail: doctortom11@126.com

Source of support: This project was supported by the National 863 project funded (grant no. 2011AA02A109)

Background: As a common malignant tumor, gastric carcinoma requires early diagnosis to improve treatment efficacy. MicroRNA (miR) molecules have highly conserved nucleotide sequences and can negatively regulate target gene expression at the translational level. miR-421 has been suggested to be related with gastric cancer occurrence. The gene polymorphism of miR-421, however, has not been reported. This study thus investigated the G/C polymorphism of miR-421 and its role in progression and prognosis of gastric cancer.

Material/Methods: A total of 96 gastric cancer patients were recruited in this study and tumor samples were collected from surgical resection. Single-nucleotide polymorphism (SNP) of miR-421 was determined by DNA sequencing for analyzing the correlation between lymph node metastasis and miR-421 genotypes. Logistic regression analysis was used to determine the relationship between genotype and risk factors of gastric cancer. Kaplan-Meier survival analysis was also performed to compare GG and GC carriers.

Results: Differential expression patterns existed between gastric cancer tissues and normal gastric mucosa. Logistic regression analysis showed GC and GG genotypes were risk factors for gastric cancer. Patients with lymph node metastasis had higher GG genotype frequency compared to those without metastasis. In survival analysis, GG carriers had shorter survival time than GC carriers. Furthermore, GG genotype was correlated with tumor prognosis ($p < 0.05$).

Conclusions: G allele of miR-421 is a risk factor for gastric cancer. GG genotype is correlated with lymph node metastasis and prognosis, indicating it is a risk factor for gastric cancer.

MeSH Keywords: **Gastric Acid • Microradiography • Transcription Factor TFIIIA**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/895652>

1703

3

1

23

Background

As the most common malignant tumor in the gastrointestinal tract, gastric carcinoma is the second leading cancer in China, with an incidence of 400,000/per year (approximately 42% of worldwide incidence). Although diagnostic and treatment technology has progressed, the prognosis of gastric cancer is still unfavorable, mainly due to the occurrence of lymph node or distal metastasis by the time of first diagnosis, making radical resection impractical. Moreover, the predictive power of prognosis using currently available methods is not high enough. Early diagnosis and accurate prediction of prognosis is, therefore, of critical importance for improving treatment efficacy [1,2]. MicroRNA (miR) is a type of single-stranded non-coding RNA molecule 22 nucleotides in length. It can inhibit or down-regulate the target gene translation by specific binding to the 3'-untranslated region (3'-UTR). It is now commonly believed that miR plays a vital role in both tumor pathogenesis and tumor suppression. Various miR molecules have been found to be related with gastric carcinoma, thus improving the diagnostic and treatment efficacy [3,4]. Studies have revealed the existence of a gene polymorphism locus at +60 of miR-421. This locus is within the seeding sequence and can regulate the expression of miR-421, suggesting a close correlation between up-regulation of miR-421 and gastric cancer occurrence [5]. The relationship between miR-421 polymorphism and gastric cancer, however, remains unclear. To further explore this correlation, the present study investigated the distribution of miR-421 genotypes and its role in occurrence of gastric cancer, in an attempt to strengthen target gene prediction.

Material and Methods

Patient information and sample collection

A total of 96 gastric carcinoma samples were collected from surgical resection of patients from January 2010 to January 2013 in the Affiliated Hospital, Academy of Military Medical Sciences. All patients were diagnosed with primary gastric carcinoma at the first tumor onset. No radio-/chemo- or molecular target therapy had been performed before the surgery. Among the 96 patients, there were 74 males and 22 females, ages 42 to 78 years old (average age=61.34 years). According to Lauren type, there were 68 cases of intestine type carcinoma and 28 cases of diffused cancer. The number of cases with high, moderate, and low differentiation was 12, 46, and 38, respectively. Using the UICC staging system, there were 38 cases of phase I+II and 58 cases of phase III+IV. The depth of infiltration was T1+T2 in 54 cases and T3+T4 in 42 cases. The locations of tumors were the gastric antrum in 38 cases, the body in 16 cases, the cardia in 21 cases, and the whole stomach in 21 cases.

This study was pre-approved by the Ethics Committee of the Affiliated Hospital, Academy of Military Medical Sciences, and we obtained written consent from all participants in accordance with the Declaration of Helsinki.

Tissue samples were collected during the surgery and frozen at -80°C . RNA was extracted before the experiment. Pathological typing of tumor tissues was performed to evaluate the infiltration degree based on the UICC standard [6]. The diagnosis of lymph node differentiation and metastasis was performed according to WHO guidelines [7]. Post-operative follow-up was performed on all patients once per month for a maximum duration of 5.5 years (average=3.6 years).

RNA extraction and qPCR analysis

Total RNA was extracted by use of an RNA extraction kit (Sigma, USA) from all gastric cancer tissues. The relative expression level of miR-421 was determined by TaqMan miRNA assay kit (Santa Cruz, USA) using RNU6 as the internal reference in 2-step reactions. In brief, cDNA was synthesized using stem-loop primers in an *in vitro* reverse transcription system using 10 ng RNA as the template (2 ng/ μL). The gradient denaturing reaction was performed as follows: 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, ending with 72°C for 7 min. In the second step, miR-421-specific primers (Forward, 5'-cacag uaggc cucaa auguu uguug auga-3'; Reverse: 5'-guguc guccg gguuu acaga caacu acu-3') were added into a 2 $\times$ PCR Taqman Universal mixture. PCR parameters were: 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, repeated for 40 cycles, ending with 72°C for 7 min. The reaction was quantified using fluorescent real-time quantitative PCR equipment (Rotor-gene, Australia). The relative level of miR-421 was determined by $2^{\Delta\text{Ct}}$ method ($\Delta\text{Ct}=\text{Ct}_{\text{miR421}}-\text{Ct}_{\text{RNU6}}$).

Statistical analysis

All collected data were processed using the SPSS 21.0 software package. Pearson chi square analysis was used to determine if genotype distribution fit Hardy-Weinberg equilibrium. Data that fit the normal distribution are presented as mean $\pm$ standard deviation (SD). The comparison of ratios was done by chi square analysis. Analysis of variance (ANOVA) was used to compare the differences in genotype frequencies of miR-421, with SNK correction coefficient for between-group comparison. The paired *t* test was used to compare the differential expression of miR-421 between tumor and normal tissues. Logistic regression analysis detected if GG/GC genotypes were risk factors for gastric cancer. A Kaplan-Meier survival curve was plotted to analyze the postoperative survival of patients. All tests were 2-tailed. The Mann-Whitney U test was used to compare nonparametric data. Statistical significance was defined when $p<0.05$.

Table 1. Clinical features of all patients.

Index	Gastric cancer	Control	t/ χ^2	P value
Sex				
Male	74	63	0.86	0.32
Female	22	15		
Age (years)				
>60	46	38	0.53	0.75
≤60	50	40		
Liver function				
ALT (U/L)	106.48±72.16	21.47±0.23	8.63	0.00
AST (U/L)	99.86±60.31	19.17±4.96	7.24	0.00
Creatinine (μmol/L)	169.45±35.69	42.36±9.68	6.72	0.00
Genotype frequency				
GG	45 (46.88%)	6 (7.69%)	7.45	0.00
GC	28 (29.17%)	10 (12.82%)		

Results

Clinical features of patients

Demographic information, such as age and sex distribution, was not significantly different between gastric cancer and control individuals ($p>0.05$, Table 1). The liver and kidney functions, however, were significantly compromised in gastric cancer patients ($p<0.05$, Table 1). A preliminary survey of genotype (GG and GC) distribution showed the existence of Hardy-Weinberg equilibrium.

Demographic and clinical features of lymph node metastasis

In gastric cancer patients with lymph node metastasis, the frequency of GG genotype of miR-421 was higher than that in patients without lymph node metastasis, suggesting that the GG genotype might be a risk factor for lymph node metastasis. Moreover, age and liver/kidney dysfunctions were shown to be major risk factors for the metastasis of gastric cancer (Table 2).

Correlation between genotypes and cancer metastasis

The frequency of GG and GC genotypes in gastric cancer patients were significantly higher than those in the control group (46.88% vs. 7.69%; 29.17% vs. 12.82%), suggesting that both genotypes are risk factors for gastric cancer. Logistic regression analysis showed that OR values of GC and GG genotypes were 2.998 (95% CI: 1.176–7.639) and 2.936 (95% CI: 1.198–7.246), respectively. The occurrence of gastric cancer was increased by 16% in GC carriers and by 28% in GG carriers. When we checked tumor metastasis, OR values of GC and

GG genotypes were 2.936 (95% CI: 0.576–1.896) and 1.846 (95% CI: 1.148–2.965), respectively (Table 3).

Prognosis and GG genotype frequency

The average relative expression level of miR-421 in all 96 gastric carcinoma tissues was 1.436 (95% CI: 0.323–0.534). There were 42 cases with higher than average expression values and 54 cases had miR-421 below the average value. When comparing the prognosis of those 2 groups of patients, we found that those with high expression levels had a median survival time of 37.34 months, while miR-421 low-expression patients had a median survival time of 54.23 months. Therefore, a relationship existed between disease prognosis and GG genotype frequency ($r=5.16$, $p<0.05$) but not GC genotype ($r=2.86$, $p>0.05$, Figure 1).

Discussion

Due to the insidious onset of gastric cancer at early stage, most patients are already at the late or terminal stage by the time of first diagnosis, causing unfavorable prognosis and lower survival rate for patients with gastric cancer [8]. miRNA can exert pluripotent biological functions via modulating multiple target gene expressions, and is thus a popular research focus in oncology [9,10]. With the advancement of bioinformatics, miRNA has been found to be related to tumor growth, development, proliferation, invasion, and apoptosis. The expression spectrum of miRNA also determines the genotype of tumor cells and may play a vital role in subtyping of tumors and prediction of metastasis, especially for the post-operative survival time and prognosis, as suggested by previous studies

Table 2. General features in metastatic patients.

Index	Lymph node metastasis (N=20)	Non-lymph node metastasis (N=64)	t/ χ^2	P value
Sex				
Male	15	51	0.69	0.52
Female	5	15		
Age (years)				
>60	16	39	3.64	0.01
≤60	4	27		
Liver function				
ALT (U/L)	146.45±70.16	48.47±17.26	9.66	0.00
AST (U/L)	156.86±80.31	68.17±12.31	8.24	0.00
Creatinine (μmol/L)	287.45±135.65	69.36±14.65	7.76	0.00
Genotype frequency				
GG	10 (50.00%)	18 (27.27%)	2.83	0.03
GC	7 (35.00%)	12 (18.18%)		

Table 3. Risk factors between genotype and gastric cancer occurrence/metastasis.

Index	β	SE	χ^2	P value	OR	95%CI
GG carriers						
Occurrence	1.098	0.465	5.289	0.023	2.998	1.176–7.639
Lymph node metastasis	0.032	0.295	0.012	0.014	1.036	0.576–1.896
GC carriers						
Occurrence	1.076	0.463	5.498	0.019	2.936	1.198–7.246
Lymph node metastasis	0.615	0.247	6.256	0.015	1.846	1.148–2.965

Figure 1. Survival curve of miR-421 GG genotype (A) and GC genotype (B) carriers.

showing the relationship between miRNA expressional level and tumor prognosis [11,12].

In various gastrointestinal tumors (e.g., gastric and colon cancer), metastatic lymph node ratio is reported to be an important prognostic factor [13,14]. In recent years, high-throughput analysis has identified miR-421 as a gastric cancer-related molecule. As an important family of human iRNA-200, the miR-421 coding gene is located on human chromosome 1p34.23, where multiple tumor-related genes have been identified [15–18]. A close correlation exists between up-regulation of miR-421 and gastric cancer occurrence, because single-nucleotide polymorphism (SNP) at certain locations causes higher risk of disease [19]. Certain SNP *loci* have been suggested to exist at the +60 locus of the miR-421 coding sequence. This locus localizes within the seed-ing sequence of miR-421, and its expression can be regulated [20–23]. The relationship between miR-421 polymorphism and gastric cancer, however, has not been reported. This study thus investigated the correlation between gastric occurrence/prognosis and G/C polymorphism within the miR-421 gene, in an attempt to further illustrate the role of miR-421 in gastric cancer.

We performed an analysis using sequencing technique to determine the alleles involved in SNP of miR-421 from tumor samples obtained from surgical resection. Our results showed differential distribution of miR-421 genotypes across tumor

and normal tissues. Multi-variant logistic regression analysis showed that both GC and GG genotypes may be risk factors for gastric cancer pathogenesis. GG genotype carriers had higher incidence of lymph node metastasis, suggesting it may be a risk factor for metastasis. Age and liver/kidney dysfunctions are also identified as major risk factors for tumor metastasis. GG carriers had significantly higher incidence of gastric carcinoma occurrence and lymph node metastasis. In a further survival analysis, we found that GG carriers had shorter survival time compared to GC genotype carriers. Therefore, miR-421 polymorphism is a factor influencing cancer prognosis.

Conclusions

This study demonstrated the possible involvement of miR-421 GG-genotype in the invasion and metastasis of gastric carcinoma, indicating it might be a potential drug target. We also revealed a correlation between miR-421 polymorphism and gastric cancer, suggesting the potential facilitation of gastric cancer pathogenesis by GG genotype via up-regulating miR-421 expression. However, this study has certain limitations due to the relatively small samples size and lack of long-term follow-up. We plan to perform a prospective study to obtain more clinical information. We believe that more miR-related modulatory mechanisms could be identified, thus providing novel methods for diagnosis, treatment, and prognostic prediction of gastric cancer.

References:

- Min H, Yoon S: Got target? Computational methods for microRNA target prediction and their extension. *Exp Mol Med*, 2010; 42(4): 233–44
- Valeri N, Gasparini P, Fabbri M et al: Modulation of mismatch repair and genomic stability by miR-155. *Proc Natl Acad Sci USA*, 2010; 107(15): 6982–87
- Fassi Fehri L, Koch M, Belogolova E et al: *Helicobacter pylori* induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS One*, 2010; 5(3): e9500
- Brandl A, Wittmann J, Jack HM: A facile method to increase titers of miRNA-encoding retroviruses by inhibition of the RNaseIII enzyme Drosha. *Eur J Immunol*, 2011; 41(2): 549–51
- Zhang X, Cui L, Ye G et al: Gastric juice microRNA-421 is a new biomarker for screening gastric cancer. *Tumour Biol*, 2012; 33(6): 2349–55
- Xu J, Qin X, Wang J et al: Chinese guidelines for the diagnosis and comprehensive treatment of hepatic metastasis of colorectal cancer. *J Cancer Res Clin Oncol*, 2011; 137(9): 1379–96
- Grávalos Castro C, Maurel Santasusana J, Rivera Herrero F et al: SEOM clinical guidelines for the adjuvant treatment of colorectal cancer. *Clin Transl Oncol*, 2010; 12(11): 724–28
- Du Y, Xu Y, Ding L et al: Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. *J Gastroenterol*, 2009; 44(6): 556–61
- Ueda T, Volinia S, Okumura H et al: Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol*, 2010; 11(2): 136–46
- Lee JW, Park YA, Choi JJ et al: The expression of the miRNA-200 family in endometrial endometrioid carcinoma. *Gynecol Oncol*, 2011; 120(1): 56–62
- Bockmeyer CL, Christgen M, Müller M et al: MicroRNA profiles of healthy basal and luminal mammary epithelial cells are distinct and reflected in different breast cancer subtypes. *Breast Cancer Res Treat*, 2011; 130(3): 735–45
- Sun T, Wang C, Xing J, Wu D: miR-429 modulates the expression of c-myc in human gastric carcinoma cells. *Eur J Cancer*, 2011; 47(17): 2552–59
- Isik A, Peker K, Firat D et al: Importance of metastatic lymph node ratio in non-metastatic, lymph node-invaded colon cancer: a clinical trial. *Med Sci Monit*, 2014; 20: 1369–75
- Isik A, Okan I, Firat D et al: A new prognostic strategy for gastric carcinoma: albumin level and metastatic lymph node ratio. *Minerva Chir*, 2014; 69(3): 147–53
- Mees ST, Mardin WA, Wendel C et al: EP300 – a miRNA-regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas. *Int J Cancer*, 2010; 126(1): 114–24
- Chen L, Tang Y, Wang J et al: miR-421 induces cell proliferation and apoptosis resistance in human nasopharyngeal carcinoma via downregulation of FOXO4. *Biochem Biophys Res Commun*, 2013; 435(4): 745–50
- Ouyang G, Wang Z, Fang X et al: Molecular signaling of the epithelial to mesenchymal transition in generating and maintaining cancer stem cells. *Cell Mol Life Sci*, 2010; 67(15): 2605–18
- Zhang Y, Li M, Wang H et al: Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg*, 2009; 33(4): 698–709
- Wu J, Li G, Yao Y et al: MicroRNA-421 is a new potential diagnosis biomarker with higher sensitivity and specificity than carcinoembryonic antigen and cancer antigen 125 in gastric cancer. *Biomarkers*, 2015; 20(1): 58–63
- Siva AC, Nelson LJ, Fleischer CL et al: Molecular assays for the detection of microRNAs in prostate cancer. *Mol Cancer*, 2009; 8: 17
- Ohashi S, Natsuizaka M, Naganuma S et al: A NOTCH3-mediated squamous cell differentiation program limits expansion of EMT-competent cells that express the ZEB transcription factors. *Cancer Res*, 2011; 71(21): 6836–47
- Jiang Z, Guo J, Xiao B et al: Increased expression of miR-421 in human gastric carcinoma and its clinical association. *J Gastroenterol*, 2010; 45(1): 17–23
- Mitchell PS, Parkin RK, Kroh EM et al: Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*, 2008; 105(30): 10513–18