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Original Article

DROSHA rs10719 T>C is associated with lymph node metastasis and clinical stage of gastric cancer patients

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Abstract: It has been proved that polymorphisms in *DROSHA* are related to the risk and outcomes of several cancers. In our study, 97 patients with stage I-III gastric cancer treated with radical gastrectomy and adjuvant chemotherapy of oxaliplatin and fluoropyrimidines were analyzed. MassARRAY MALDI-TOF system was used to determine the genotypes. The 2-year DFS rate was 60.8% and the 3-year OS rate was 73.8%. In dominant model, we found that rs10719 TC+CC genotype carriers were less likely to develop lymph node metastasis (P=0.031). Compared with TC+CC genotype carriers, more patients with TT genotype were in stage III (P=0.021). The 3-year OS was significantly different for patients with or without lymph node metastasis (89.3% vs 63.3%, P=0.013) and for patients with stage I-III disease (100.0%, 88.6% and 55.8%, P=0.015). After the multi-variants' cox regression analysis, lymph node status (P=0.014, RR: 9.556, 95% CI: 1.586-57.590) was found to be an independent prognostic factor for these patients. These results suggested that *DROSHA* rs10719 T>C may be associated with lymph node metastasis and clinical stage of gastric cancer in a Chinese population.

Keywords: *DROSHA*, polymorphism, gastric cancer, prognosis

Introduction

Although the incidence of gastric cancer has been declining in some developed countries, it is still much higher in developing countries, especially in China. By some estimation, gastric cancer is the fifth most common cancer and the third leading cause of cancer death worldwide [1]. Despite the advance of diagnosis and treatment during the past decades, the prognosis for gastric cancer patients remains poor. Once patients develop recurrence or metastasis, the median overall survival time is usually less than 12 months [2]. Therefore, it is very important to look for tumor markers which can predict tumor invasion and prognosis.

MicroRNAs (miRNAs) are 21- to 24-nucleotide-long, highly conserved, single-strand, non-coding RNAs that regulate gene expression through base pairing with target mRNAs at the 3'-untranslated region, leading to mRNA cleavage or translational repression [3, 4]. It has been proved that miRNA can serve as biomarkers for diagnosis, therapeutic targets and prognosis in cancer patients [5-8]. MiRNA biogenesis begins in the nuclease. Related genes are transcripted to create the long primary miRNAs (pri-miRNAs) by RNA polymerase II, which are then cut into ~70 nt hairpin precursors (pre-miRNAs) by RNase III DROSHA and its cofactor. These precursors are exported to the cytoplasm by EXPORTIN-5 and are further diced into ~22 nt miRNA duplexes by RNase III DICER. MiRNA duplexes are assembled into the miRNA-induced silencing complex (miRISC) which guides the mature miRNAs to their target mRNAs [9-13]. Polymorphisms in miRNA processing machinery genes such as *DROSHA*, *DICER* can modulate their expression and then influence the expression and function of miRNAs [14, 15]. Up to now, some researchers have demonstrated that polymorphisms in *DROSHA*, *DICER* and *XPO5* are associated with the risk or prognosis of cancers such as bladder cancer [15], colorectal cancer [16] and renal cancer [17]. However, reports about polymorphisms in *DROSHA* and gastric cancer are rare. Since DROSHA is an important nuclease that exe-

cutes the initial step in miRNA processing, we enrolled 97 stage IB-III gastric cancer patients who received gastrectomy followed by oxaliplatin and fluoropyrimidines as adjuvant chemotherapy, and explored the association between *DROSHA* rs10719 T>C and the clinical characteristics and outcomes of these patients.

Materials and methods

Subjects

97 patients who were histopathologically diagnosed with gastric cancer and treated in Jiangxi Cancer hospital between January 2011 and May 2013 were retrospectively included. This study was approved by the Ethics Committee of Jiangxi Cancer Hospital.

Inclusion criteria

Ethnic Chinese, male or female, at least 18 years old; Histologically diagnosed with gastric adenocarcinoma; According to American Joint Committee on Cancer Staging classification (7th ed., 2010), patients were proved to be at stage IB-III; Eastern Cooperative Oncology Group (ECOG) score was between 0 and 2 points; Patients received radical gastrectomy and adjuvant chemotherapy of oxaliplatin and fluoropyrimidines; Neutrophil counts of 1.5×10^9/L or higher and platelets of 80×10^9/L or higher; alanine aminotransferase and aspartate aminotransferase levels ≤2.5 times the upper limit of normal value; a total bilirubin level ≤1.5 times the upper limit of normal value; measured or calculated creatinine clearance of >60 ml/min; and a normal electrocardiogram; The expected survival time was 3 months or more; Patients gave written informed consent and had to be accessible for treatment and follow-up.

Exclusion criteria

The presence of contraindications to gastrectomy; The presence of contraindications to chemotherapy; The presence of second primary tumors; Pregnant and lactating women.

Treatment regimens and follow-up visit

Chemotherapy regimens included 1) 61 patients received mFOLFOX6: oxaliplatin 85 mg/m^2 intravenous (IV) over 2 hours on day 1+ leucovorin 400 mg/m^2 IV over 2 hours on day 1+5-FU 400 mg/m^2 IV bolus on day 1, 5-FU 2,400 mg/m^2 continuous intravenous infusion 46 hours, every 2×24 weeks; 2) 22 patients received oxaliplatin 130 mg/m^2 IV over 2 hours on day 1+ leucovorin 200 mg/m^2 IV over 2 hours on day 1-4+5-FU 600 mg/m^2 IV on day 1-4, every 3×24 weeks; 3) eight patients received XELOX: oxaliplatin 130 mg/m^2 IV over 2 hours on day 1+ capecitabine 1,000 mg/m^2 twice daily on day 1-14, every 3×24 weeks; 4) six patients received SOX: oxaliplatin 130 mg/m^2 IV over 2 hours on day 1+ S-1 40-60 mg twice daily on day 1-14, every 3×24 weeks.

Demographic information and clinical characteristics were collected from each patient via clinical record. Data on whether and when a patient had relapsed or died were obtained from inpatient and outpatient records, patient or family contact. The DFS (disease-free survival) is defined as the time from the diagnosis to tumor relapse or metastasis, and the OS (overall survival) is defined as the time from the diagnosis until death caused by any reason.

DNA extraction and genotyping

A 5 ml peripheral blood sample was collected from each patient. Genomic DNA was isolated by standard proteinase K digestion and phenol-chloroform extraction from the blood samples. DNA purity and concentration were determined by spectrophotometric absorbance measurements at 260 and 280 nm using an ultraviolet spectrophotometer.

MassARRAY MALDI-TOF System (Sequenom Inc., San Diego, CA, USA) was used for genotyping by the method described in Sequenom Genotyping Protocol. The PCR primers and probes were designed according to the reference sequences in NCBI GenBank database. Primers were 5'-ACGTTGGATGACAATAGCGATTTGACTCT G-3' (sense) and 5'-ACGTTGGATGGAGACCTAGCCTAGTTTTCC-3' (antisense). Duplicate samples and negative controls (without DNA) were set for quality assurance of genotyping. Concordance for duplicate samples was 100% for all assays.

Statistical analysis

The statistical analysis was performed with the SPSS version 18.0 software package (SPSS Inc, Chicago, IL, USA). The descriptive analysis

Table 1. Rs10719 genotypes and clinicopathological features

Variables	Codominant model				Dominant model			Recessive model		
	TT	TC	CC	P	TT	TC+CC	P	TT+TC	CC	P
Age (years)										
<60	20	39	9		20	48		59	9	
≥60	12	13	4	0.458	12	17	0.251	25	4	1.000
Sex										
Male	20	40	8		20	48		60	8	
Female	12	12	5	0.288	12	17	0.251	24	5	0.469
Location										
Non-cardia	29	49	11		29	60		78	11	
Cardia	3	3	2	0.389	3	5	1.000	6	2	0.291
Differentiation										
Well to moderate	9	12	5		9	17		21	5	
Poor/signet-ring	23	40	8	0.523	23	48	0.837	63	8	0.308
Depth of invasion										
T1-2	6	11	2		6	13		17	2	
T3-4	26	41	11	1.000	26	52	0.884	67	11	1.000
LN metastasis										
N0	5	19	5		5	24		24	5	
N+	27	33	8	0.097	27	41	0.031	60	8	0.469
Clinical stage										
I-II	7	25	5		7	30		32	5	
III	25	27	8	0.056	25	35	0.021	52	8	0.980

Abbreviations: LN, lymph node.

of genotypes and clinicopathological features was expressed both in absolute values and percentages. The association between the genotypes and clinicopathological features was analyzed based on chi-square or Fisher's exact probability tests. Disease-free and overall survival curves were drawn with the Kaplan-Meier product limit method for each of the different genotypes. Comparisons were made with the log-rank test. Hazard ratios of recurrence/metastasis and death with 95% confidence intervals were estimated by using the Cox model. All statistical tests are two-sided tests and $P<0.05$ was considered significant.

Results

All 97 patients were included. The median age at the time of diagnosis was 54 years (range =19-79). 68 (70.1%) patients were man. Only 8 (8.2%) patients had primary lesions located in cardia. Most patients (n=71, 73.2%) were diagnosed with poorly differentiated gastric cancer or gastric cancer with signet ring cells. There were 19 (19.6%) and 78 (80.4%) patients in T1-2 and T3-4, respectively. More patients (n=68, 70.1%) were with regional lymph node metastasis.

Relationship between genotypes and clinicopathological features

Among all patients enrolled in this study, 32 patients (67.2%) have TT genotype; 52 patients (32.8%) have TC genotype; 13 patients (32.8%) have CC genotype. TT genotype carriers were more likely to develop regional lymph node metastasis compared to TC+CC carriers ($P=0.031$). More patients with TT genotype were in stage III ($P=0.021$). There is no significant difference of other clinicopathological features among different genotypes (**Table 1**).

Relationship between genotypes and survival time

The 2-year DFS rate and 3-year OS rate for all patients enrolled were 60.8% and 73.4%, respectively. There was no significant association between rs10719 genotypes and DFS or OS.

Table 2. Rs10719 genotypes and survival

Variables	2 y DFS	*P*	3 y OS	*P*
Codominant model				
TT	47.7%		60.9%	
TC	72.9%		88.4%	
CC	44.0%	0.114	70.1%	0.295
Dominant model				
TT	47.7%		60.9%	
TC+CC	66.6%	0.102	82.9%	0.140
Recessive model				
TT+TC	63.6%		73.8%	
CC	44.0%	0.426	70.1%	0.977

Abbreviations: DFS, disease free survival; OS, overall survival.

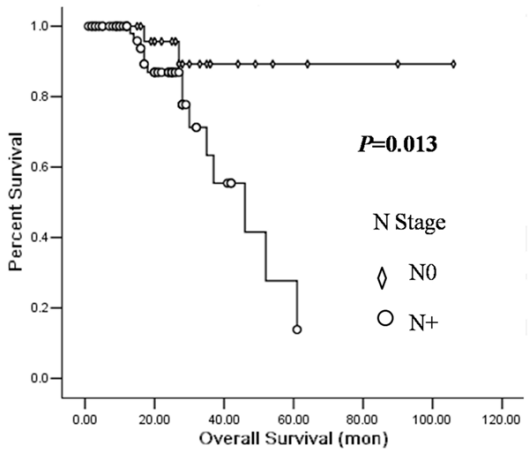

Figure 1. Kaplan-Meier graph of overall survival for patients with or without lymph node metastasis. Abbreviations: N, regional lymph nodes; N0, without lymph node metastasis; N+, with lymph node metastasis.

The 2-year DFS rates and 3-year OS rates for patients with TT, TC and CC genotypes were 47.7%, 72.9%, 44.0% (P=0.114) and 60.9%, 88.4%, 70.1% (P=0.295), respectively (**Table 2**).

We also investigated the survival rate among patients with different clinicopathological features. We found that T stage (P=0.012) and clinical stage (P=0.011) significantly affected DFS of patients. The results also indicated that age (P=0.046), N stage (P=0.013) (**Figure 1**) (Supplementary Table) and clinical stage (P=0.015) were significantly associated with OS (**Table 3**).

After the multi-variants' cox regression analysis, status of lymph node metastasis (P=0.014, RR: 9.556, 95% CI: 1.586-57.590) was found to be an independent prognostic factor for these patients.

Discussion

DROSHA is a member of RNase III superfamily and is an important nuclease that executes the initial step in miRNA processing by cutting pri-miRNA to pre-miRNA [11]. Rs10719 T>C polymorphism is located in the 3' untranslated region (UTR) of *DROSHA*. It has been proposed that some of the 3' UTR polymorphisms may interfere with miRNA function, which lead to differential gene expression and then affect the development, progression and prognosis of several kinds of cancers [18, 19]. Functional assays indicated that *DROSHA* rs10719 T to C substitution can decrease the binding activity of has-mir-27b with *DROSHA* 3' UTR, resulting in the increased level of *DROSHA* 3' UTR luciferase expression [15].

It has been reported that rs10719 T>C is associated with the risk of several cancers. Yuan et al. investigated the association between rs-10719 and the risk of bladder cancer in a Chinese population [15]. They found that rs10719 TC/CC genotype can increase the risk of bladder cancer among male patients and ever smokers, compared with TT genotype. Cho et al. investigated whether polymorphisms in miRNA machinery genes are associated with the development of colorectal cancer [16]. The results indicated that patients who diagnosed with hypertension or diabetes mellitus and carried *DROSHA* rs10719 CC genotype showed increased risk of colorectal cancer. Combined analysis suggested that *DROSHA* rs10719 CC genotype carriers and *RAN* rs14035 CC heterozygotes had reduced risk of rectal cancer.

To our knowledge, there is no report about relationship between *DROSHA* rs10719 and gastric cancer. Xie et al. found that some polymorphisms of miRNA processing machinery genes such as rs14035 from *RAN*, rs3742330 from *DICER*, and rs9623117 from *TNRC6B* were associated with the risk of gastric cancer, but rs10719 from *DROSHA* was not analyzed in their study [20]. Our study first reported that rs10719 was associated with the lymph node

Table 3. Clinicopathological features and survival

Variables	2 y DFS	*P*	3 y OS	*P*
Age (years)				
<60	64.0%		78.1%	
≥60	52.1%	0.211	61.5%	0.046
Sex				
Male	58.5%		69.0%	
Female	65.5%	0.237	85.7%	0.125
Location				
Non-cardia	59.4%		75.0%	
Cardia	75.0%	0.560	75.0%	0.864
Differentiation				
Well to moderate	44.3%		78.6%	
Poorly/signet-ring	66.2%	0.081	72.4%	0.346
Depth of invasion				
T1-2	88.2%		100.0%	
T3-4	53.5%	0.012	67.3%	0.110
LN metastasis				
N0	72.1%		89.3%	
N+	55.5%	0.120	63.3%	0.013
Clinical stage				
I	100.0%		100.0%	
II	67.8%		88.6%	
III	50.0%	0.011	55.8%	0.015
Vascular invasion				
No	58.9%		79.6%	
Yes	70.0%	0.653	44.4%	0.078
Neural invasion				
No	61.7%		78.8%	
Yes	53.0%	0.258	29.2%	0.050

Abbreviations: LN, lymph node; DFS, disease free survival; OS, overall survival.

metastasis and clinical stage of gastric cancer patients. Compared with TC+CC genotype carriers, TT genotype carriers were more likely to develop lymph node metastasis (P=0.031). More patients with TT genotype were in stage III (P=0.021). Zhang et al. in China demonstrated that DROSHA protein expression was associated with tumor grade, T stage, lymph node metastasis and TNM stage of gastric cancer [21]. DROSHA protein expression was negatively related to tumor malignancy and invasion. Higher expression level of DROSHA protein was observed in well differentiated tumours. Since Yuan's study suggested that *DROSHA* rs10719 T to C substitution might increase the DROSHA protein level, the results from Zhang's study supported our findings.

We also analyzed the association between rs10719 and survival time of these gastric cancer patients who received gastrectomy and adjuvant chemotherapy, but no significant association was observed. Up to now, data about rs10719 and prognosis of cancers are rare. Researchers in M.D. Anderson Cancer Center found that compared with the CG haplotype (in order of rs10719 and rs6877842), a 57% reduction in recurrence risk of renal cell carcinoma was observed for the CC haplotype (HR=0.43; 95% CI=0.22-0.87; P=0.02) and the HR was 0.50 (95% CI=0.27-0.90; P=0.02) for the TG haplotype [17]. In Cho's study [16], no significant association between genotype frequencies for rs10719 and colorectal cancer patient survival was observed. Li et al. [22] found that *DROSHA* rs6877842 and *DICER* rs3742330 were associated with the survival of T cell lymphoma, while *DROSHA* rs10719 was not related to the prognosis of these patients.

Researches about relationship between *DROSHA* expression level and prognosis of cancers showed inconsistent results. The survival time was shorter for nasopharyngeal [23] or ovarian cancer [24] patients with lower expression level of *DROSHA*. While for bladder cancer [15] and non-small cell lung cancer patients [25], the higher level of *DROSHA* expression indicated poorer prognosis. For breast cancer patients, no significant association between expression level of *DROSHA* and outcome of breast cancer patients was observed [26]. In Zhang's study, gastric cancer patients in DROSHA protein positive group had a higher survival rate than those in DROSHA negative group. However, no association between rs10719 and survival rate was observed in our research. One possible explanation is that the sample size of our study was relatively small to observe the difference. Secondly, rs10719 might interact with other polymorphisms laid in *DROSHA*, so studies including more polymorphisms are needed. Additionally, in Yuan's study, increased level of DROSHA 3' UTR luciferase expression was observed. Since the DROSHA mRNA level was not different among genotypes, the authors assumed that rs10791 might lead to different DROSHA protein expression. However, as they did not test the protein level, the assumption needs to be verified.

In conclusion, for gastric cancer patients who received gastrectomy and adjuvant chemotherapy, we first demonstrated that rs10719 was associated with lymph node metastasis and clinical stage. More TT genotype carriers developed lymph node metastasis and were in stage III. No relationship between genotypes and survival was observed in our study. Our results still need to be supported by larger, prospective studies. It is also necessary for us to explore the function of rs10719 to clarify the underlying mechanisms.

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Disclosure of conflict of interest

None.

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