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Original Article

PIK3C2G copy number is associated with clinical outcomes of colorectal cancer patients treated with oxaliplatin

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Abstract: Purpose: To investigate whether the copy number of PIK3C2G is associated with clinical outcomes for stage III colorectal cancer (CRC) patients treated with oxaliplatin-based chemotherapy. Methods: A total of 142 CRC patients who received first-line oxaliplatin-based chemotherapy after curative surgery in Ruijin Hospital and The Tenth People's Hospital were recruited in this study. Patients were enrolled between June 2006 and December 2011, with follow-up to January 2014. Quantitative real-time PCR method was used to detect the copy number of PIK3C2G. Cox proportional hazards model and Kaplan-Meier curves were used to analyze the association between PIK3C2G copy number and clinical outcome. Results: In patients with stage III disease, low copy number of PIK3C2G was associated with increased risk of both recurrence (HR, 2.44, 95% CI, 1.33-4.47, P=0.004) and death (HR, 2.89, 95% CI, 1.49-5.60, P=0.002). Multivariate analysis also indicated that low PIK3C2G copy number was a significant and independent predictor of OS and RFS of stage III CRC. Conclusions: PIK3C2G is capable of predicting the recurrence and overall survival of stage III CRC patients receiving oxaliplatin-based therapy.

Keywords: PIK3C2G, recurrence, overall survival, colorectal cancer, oxaliplatin

Introduction

Colorectal cancer (CRC) is now the third most common cancer in the world and the fourth most common cause of death from cancer [1]. Attributing to the advancement in surgical techniques and the popularization of chemotherapy and targeted therapy, the treatment of CRC has evolved rapidly over several decades. Oxaliplation in combination with 5-Fluorouracil (5-Fu, capecitabine) was regarded as first-line chemotherapy in both adjuvant and palliative settings for advanced CRC, but the therapeutic benefit has reached a therapeutic plateau [2, 3]. It is critical to identify novel biomarkers to allow personalized therapy in patients with CRCs.

Phosphoinositide 3-Kinase (PI3Ks) are lipid kinases that participate in the regulatory mechanism of multiple intracellular trafficking [4]. PI3K is originally identified as a heterodimeric complex consisting of an 85 KDa regulatory subunit and a 110 kDa catalytic subunit [5]. PI3K kinases target lipids to produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 via phosphorylation of the D-3 position hydroxyl group of the inositol ring of phosphatidylinositol. These phosphorylated lipids act as second messengers and involve in the regulation of diverse cellular events, including proliferation, transformation, differentiation, motility, survival and intracellular trafficking [6]. Based on their structural and substrate specificity, PI3Ks can

be divided into three different classes (class I, II and III). Class I PI3Ks including PIK3CA target PIP2 for phosphorylation to produce PIP3 which recruits PDK1 to activate downstream AKT signaling [7, 8]. Class III PI3Ks are homologous to the yeast vesicular-protein-sorting protein Vps34p which is involved in trafficking of protein and vesicles. Class II PI3Ks are the least studied kinases to date. They are larger proteins (~170 kDa) than other members of PI3Ks and defined by the presence of a carboxyl-terminal C2 domain. Class II PI3Ks are divided into three isoforms named PI3KC2A, PI3KC2B and PI3KC2G. They phosphorylate PtdIns and PtdIns(4)P, but not PtdIns(4,5)P_2 in vitro [9].

The roles of certain PIK3 family members vary substantially in cancer. PIK3CA acts as an oncogene whereas PIK3CG is deemed to be a tumor suppressor gene [10-12]. Genomic PIK3CA amplification appears in a variety of cancers including colon cancer, ovarian cancer, cervical cancer, thyroid cancer, and non-small cell lung cancer (NSCLC) and significantly associated with poor prognosis [13-18]. However, there are very few studies to investigate the association between PI3KC2G copy number and clinical outcomes of CRC patients.

In the present study, with the aim to identify a biomarker to predict the efficacy of oxaliplatin-based chemotherapy among CRC patients, we computed the relative copy number (RCN) of PIK3C2G in tumor samples from 142 stage III CRC patients treated with oxaliplatin-based chemotherapy after curative surgery and explored the possible associations of PIK3C2G copy number with pathological features as well as clinical outcomes.

Materials and methods

Patients and tissue collection

A total of 142 CRC patients were recruited from Ruijin Hospital affiliated to Shanghai Jiaotong University and The Tenth People's Hospital affiliated to Shanghai Tongji University between June 2006 and December 2011, with follow-up to January 2014. All these patients were histologically confirmed stage III adenocarcinomas according to the criteria proposed by the Standard American Joint Committee on Cancer (AJCC). Only patients receiving post-operative oxaliplatin-based chemotherapy at least of six cycles were admitted in this study. We reviewed patients' medical records to collect clinical information including date of diagnosis, performance status, clinical stage, tumor location, histologic grade, pathologic stage, treatment, and tumor recurrence. Written informed consent was obtained from all participants. This study was approved by the Medical Ethical Committee of Shanghai Jiaotong University and Tongji University.

DNA extraction

Manual macrodissection of tumor samples were referred to the protocol described previously [19]. Formalin-fixed, paraffin-embedded tissues were used for DNA extraction. 5-μm-thick sections from the block was subjected to standard deparaffinization procedures and proteinase K digestion overnight. After digestion, DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, CA, USA) according to the manufacturer's instruction.

Quantitative PCR and copy number analysis

20 ng of genomic DNA from each sample was subjected to Quantitative PCR using the SYBR Green kit (Qiagen, Germany). GAPDH was used as a reference gene. The sequences of the PCR primers for PIK3C2G and GAPDH were as follows: 5'-CATCAAGTTAGCAAAGCACC-3' (forward) and 5'-AATCTGGAATCATCAGCACC-3' (reverse) for PIK3C2G and 5'-TGGACCTGACCTGCCGTCTAGAAA-3' (forward) and 5'-GGAGGAGTGGGTGTCGCTGTTGAA-3' (reverse) for GAPDH. The thermal cycles conditions were 2 min at 95°C followed by 40 cycles at 95°C for 15 s, 60°C for 20 s, 72°C for 20 s. All the samples were run in triplicate. Data points that generated triplicate Ct values with over one cycle variance were excluded from analysis. The mean Ct value obtained from each sample was normalized to the averaged copy number of all samples. ΔΔCt was then computed by subtracting ΔCt of a target gene from ΔCt of the reference gene (GAPDH) and then subjected to analysis with the $2^{-\Delta\Delta Ct}$ method [20].

Statistical analysis

Pearson Chi-square (χ^2) was performed to compare the distributions of categorical variables. Student's t test was used to assess the difference of continuous variables. To assess the association of gene copy number with colorectal cancer survival, we dichotomized PIK3C2G copy number using a cut-off point in all patients.

Table 1. Correlation between PIK3C2G copy number and clinicopathological parameters of colorectal cancer

Clinicopathological factors	NO. of patients	PIK3C2G gene copy number		*p* value
	N=142	High N (%)	Low N (%)	
Age (years)		114	28	
≥65	56	48 (42.1%)	8 (28.6%)	0.189
<65	86	66 (57.9%)	20 (71.4%)	
Sex				
Male	86	68 (59.6%)	18 (64.3%)	0.653
Female	56	46 (40.4%)	10 (35.7%)	
Tumor site				
rectum	69	57 (50.0%)	12 (42.9%)	0.498
colon	73	57 (50.0%)	16 (57.1%)	
Histology				
Well/Moderate	116	95 (83.3%)	21 (75.0%)	0.307
Poor	26	19 (16.7%)	7 (25.0%)	
Lymph node metastasis				
≥4	31	23 (20.2%)	8 (28.6%)	0.334
<4	111	91 (79.8%)	20 (71.4%)	
TNM stage				
IIIA	13	12 (10.5%)	1 (3.6%)	0.184
IIIB	102	78 (68.4%)	24 (85.7%)	
IIIC	27	24 (21.1%)	3 (10.7%)	
Recurrence				
Yes	55	38 (33.3%)	17 (60.7%)	0.008
No	87	76 (66.7%)	11 (39.3%)	
Death				
Yes	44	29 (25.4%)	15 (53.6%)	0.004
No	98	85 (74.6%)	13 (46.4%)	

Table 2. Univariate and multivariate analyses for recurrence-free survival (Cox proportional hazards model)

Variable	univariate analysis			multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age (≥65 vs <65 year old)	1.00	0.58-1.72	0.986	0.82	0.47-1.43	0.490
Gender (Male vs Female)	0.79	0.46-1.35	0.393	1.49	0.86-2.58	0.153
Tumor site						
Rectum vs Colon	0.94	0.56-1.61	0.833	1.02	0.86-2.58	0.954
Histology						
poor vs well/moderate	1.64	0.88-3.06	0.119	1.80	0.95-3.43	0.072
Lymph node metastasis						
n≥4 vs n<4	2.30	1.31-4.05	**0.004**	1.05	0.51-2.15	0.905
TNM stage						
IIIC vs IIIA+IIIB	3.97	2.24-7.03	**<0.001**	4.91	2.35-10.24	**<0.001**
PIK3C2G (low vs high)	1.91	1.08-3.39	**0.024**	2.44	1.33-4.47	**0.004**

Bold items highlight P<0.05. HR: hazard ratio; CI: confidence interval; RFS: recurrence-free survival.

The study endpoints were overall survival (OS) and recurrence-free survival (RFS). OS was calculated from pathologic diagnosis to death, regardless of cause. RFS was defined as the time from pathologic diagnosis till first disease recurrence or metastasis or death from any cause, whichever occurred first. Survival curves were generated using the Kaplan-Meier method, with significance evaluated using the Mantel-Cox log-rank test. The Cox proportional hazards model was used to estimate hazard ratios (HRs) of clinical outcomes adjusted by baseline patient variables. Statistical analyses were done by Statistical Program for Social Sciences (SPSS) 20.0 software (Chicago, IL, United States). P<0.05 was considered statistically significant.

Results

Patient characteristics

A total of 142 colorectal carcinoma patients were enrolled in this study. The median follow-up time was 45 months. 55 patients (38.7%) exhibited tumor recurrence and 44 patients (31%) died. 9.2% of 142 (n=13) patients were stage IIIA, 71.8% (n=102) were stage IIIB, 19% (n=27) were stage IIIC. The clinicopathological features of the study population were summarized in **Table 1**.

Correlation analysis of PIK3C2G copy number with Clinicopathological features

To investigate the clinical relevance of RCN of PIK3C2G in CRC, patients were dichotomized into high copy number group (RCN≥0.509, n=114) and low copy number group (RCN<

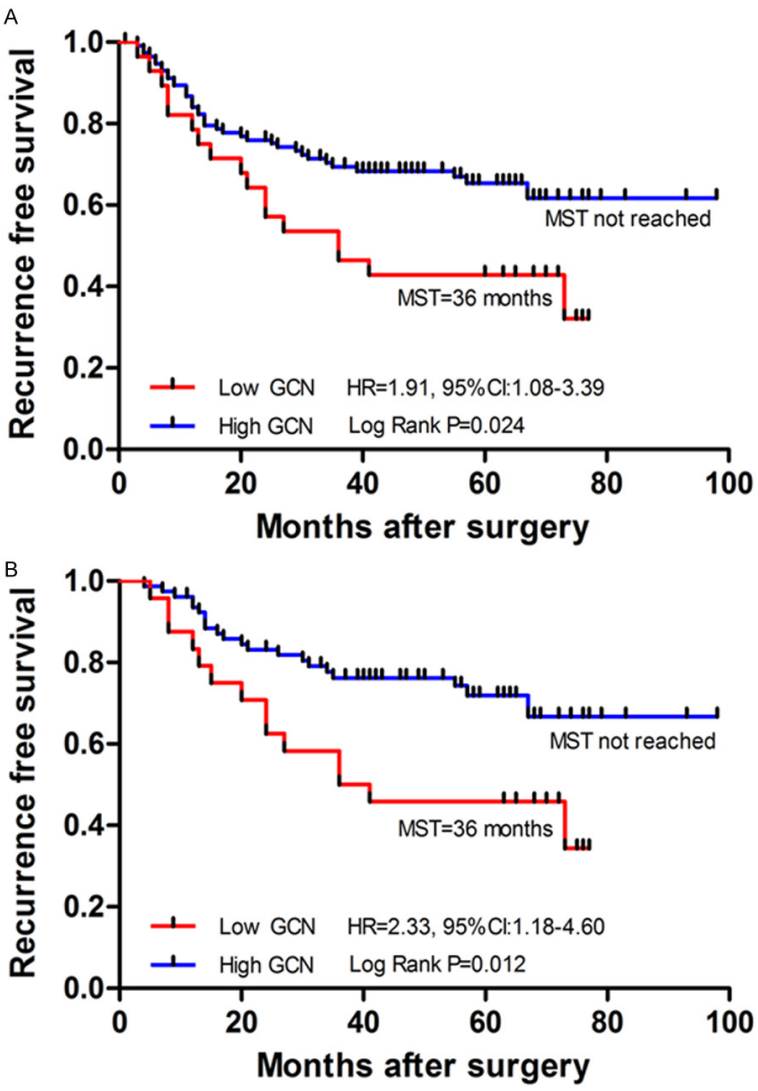

Figure 1. Kaplan-Meier survival curves of patients with stage III and IIIB disease receiving oxaliplatin-based chemotherapy. A. RFS in stage III patients. B. RFS in stage IIIB patients. MST: medial survival time.

0.509, n=28) using a quartile cut-off (RCN= 0.509). Correlation between the copy number variation of PIK3C2G and clinicopathological parameters were examined. Our results showed that the copy number of PIK3C2G was not significantly associated with examined clinicopathological parameters, such as age (P= 0.189), gender (P=0.653), tumor site (P=0.498), histology grade (P=0.307), lymph node metastasis (P=0.334) and TNM stage (P=0.184) (**Table 1**).

PIK3C2G copy number is significantly associated with recurrence of stage III CRC patients

Univariate analysis showed that recurrence of CRC patients was significantly associated with lymph node metastasis (HR, 2.30, 95% CI, 1.31-4.05, P= 0.004), poor differentiation (HR, 3.97, 95% CI, 2.24-7.03, P<0.001) and low copy number of PIK3C2G (HR, 1.91, 95%CI, 1.08-3.39, P=0.024) (**Table 2**). In the multivariate analysis adjusting for age, gender, tumor site, histology grade, lymph node metastasis and TNM stage, the patients with low copy number of PIK3C2G were shown to have a 2.44-fold increased risk of recurrence with high significance (P=0.004) (**Table 2**). The Kaplan-Meier curve showed patients with high copy number had a better RFS (log-rank, P=0.024) than those with low copy number (**Figure 1A**). Moreover, we performed association analysis between PIK3C2G copy number and recurrence among IIIB patients. It was found that low copy number of PIK3C2G was significantly associated with recurrence (HR, 2.33, 95% CI, 1.18-4.60, log-rank, P=0.012) (**Figure 1B**).

PIK3C2G copy number is significantly associated with survival of stage III CRC patients

With regard to overall survival, univariate analysis showed that death was highly correlated with lymph node metastasis (HR, 2.85, 95% CI, 1.55-5.23, P=0.001), TNM stage (HR, 3.73, 95% CI, 1.98-7.02, P<0.001), poor differentiation (HR, 2.39 95% CI, 1.25-4.57, P=0.009) and low copy number of PIK3C2G (HR, 2.13, 95% CI, 1.14-3.97, P= 0.015) (**Table 3**). Multivariate analysis revealed that death was significantly associated with poor differentiation (HR, 2.61, 95% CI, 1.31-5.20, P=0.006), TNM stage (HR, 4.42, 95% CI, 2.01-9.72, P<0.001) and low copy number of PIK3C2G (HR, 2.89, 95% CI, 1.49-5.60, P=0.002) (**Table 3**). The Kaplan-Meier curve showed that patients with low copy number had a worse survival (log rank, P=0.015) (**Figure 2A**). Additionally, association analysis was performed between PIK3C2G copy number and

Table 3. Univariate and multivivariate analyses for overall survival (Cox proportional hazards model)

Variable	univariate analysis			multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age (≥65 vs <65 year old)	1.21	0.66-2.19	0.537	0.62	0.33-1.14	0.122
Gender (Male vs Female)	0.79	0.43-1.43	0.430	1.65	0.88-3.09	0.119
Tumor site						
Rectum vs Colon	0.81	0.45-1.47	0.492	1.15	0.63-2.11	0.650
Histology						
poor vs well/moderate	2.39	1.25-4.57	**0.009**	2.61	1.31-5.20	**0.006**
Lymph node metastasis						
n≥4 vs n<4	2.85	1.55-5.23	**0.001**	0.80	0.38-1.68	0.557
TNM stage						
IIIC vs IIIA+IIIB	3.73	1.98-7.02	**<0.001**	4.42	2.01-9.72	**<0.001**
PIK3C2G (low vs high)	2.13	1.14-3.97	**0.015**	2.89	1.49-5.60	**0.002**

Bold items highlight P<0.05. HR: hazard ratio; CI: confidence interva; OS: overall survival.

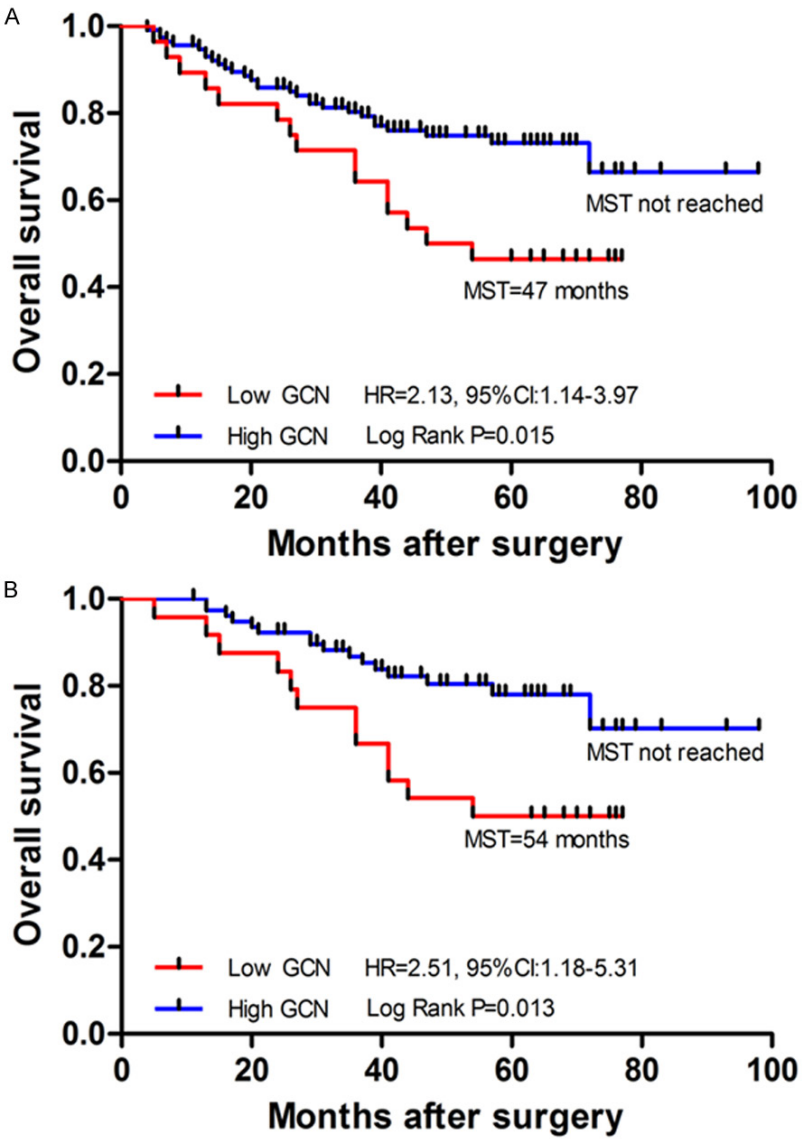

Figure 2. Kaplan-Meier survival curves of patients with stage III and IIIB disease receiving oxaliplatin-based chemotherapy. A. OS in stage III patients. B. OS in stage IIIB patients. MST: medial survival time.

overall survival among IIIB patients. It demonstrated that patients with low copy number of PIK3C2G had a 2.51-fold increased risk of death with high significance (P= 0.013) (**Figure 2B**).

Discussion

Oxaliplatin-based chemotherapy has long been recommended for stages III CRC patients, however the response to chemotherapeutic agents in patients varies greatly [3]. Till now, tumor-node-metastasis (TNM) system remains the most commonly used criteria to predict prognosis and identify the need for chemotherapy in patients with CRC [21]. However, response rate of colorectal cancer patients to oxaliplatin-based drugs is less than 50% [22, 23]. Consequently, a substantial portion of patients do not benefit from oxaliplatin-based chemotherapy and have to suffer from the side effects [24]. Hence, it is important to identify subgroups of patients who would benefit from chemotherapy, which may allow personalized treatment to improve the efficacy and reduce the risk of adverse effect. To our knowledge, this is the first study to investigate the association of copy number of PIK3C2G with

clinical outcomes of stage III CRC receiving oxalipaltin-based chemotherapy. Our results demonstrated PIK3C2G copy number was greatly associated with clinical outcomes of stage III CRC patients.

In our study, we found that low copy number of PIK3C2G was significantly associated with poor clinical outcomes in stage III CRC with radical resection. Remarkably, multivariate analysis showed that this association was independent of age, gender, tumor site, thereby confirming that PIK3C2G was a novel biomarker to predict recurrence and overall survival of colorectal cancer patients treated with oxaliplatin-based therapy.

Although conventional TNM staging system revealed stage IIIC CRC patients had an increased risk of recurrence and death compared to IIIA and IIIB, it was not able to predict the risk in a specific subgroup of CRC like IIIB (**Tables 2**, **3**). Similarly, histology grade was effective to predict the risk of death between poor and well differentiation patients (**Table 3**). However, it was not capable of predicting the risk in a subgroup of patients such as poor or well differentiation patients. The data presented here showed that PIK3C2G copy number was able to predict both recurrence and overall survival not only among stage III CRC patients but also in a specific subgroup of stage IIIB patients (**Figure 2A**, **2B**). The sample size of stage IIIA (n=13) and IIIC (n=27) patients were too small to analyze and a larger patient pool of stage IIIA and IIIC was required for further analysis.

So far, little is known about the role of PIK3C2G in colon cancer. Contrary to the class I PI3K subfamily member PIK3CA, another class I PI3K member PIK3CG was found to be down-regulated in colon cancer and its low expression was significantly associated with invasion and differentiation of colon cancer [12]. Our findings also showed that patients with low copy number of PIK3C2G had a higher ratio of poor differentiation than patients with high copy number (**Table 1**). It indicates that PIK3C2G could have a similar function to PIK3CG involving in progression of colon cancer.

Conclusion

In conclusion, PIK3C2G is capable of predicting the recurrence and overall survival of stage III CRC patients receiving oxaliplatin-based therapy.

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Disclosure of conflict of interest

None.

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