

RESEARCH ARTICLE

Genetic Variations in the *HIF1A* Gene Modulate Response to Adjuvant Chemotherapy after Surgery in Patients with Colorectal Cancer

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Abstract

Background: Hypoxia-inducible factor 1 α (HIF-1 α) plays an important role in regulating cell survival and angiogenesis, which are critical for tumor growth and metastasis. Genetic variations of *HIF1A* have been shown to influence the susceptibility to many kinds of human tumors. Increased expression of HIF-1 α has also been demonstrated to be involved in tumor progression. However, the prognostic value of single nucleotide polymorphisms (SNPs) in the *HIF1A* gene remains to be determined in most cancer types, including colorectal cancer (CRC). In this study, we sought to investigate the predictive role of *HIF1A* SNPs in prognosis of CRC patients and efficacy of chemotherapy. **Materials and Methods:** We genotyped two functional SNPs in *HIF1A* gene using the Sequenom iPLEX genotyping system and then assessed their associations with clinicopathological parameters and clinical outcomes of 697 CRC patients receiving radical surgery using Cox logistic regression model and Kaplan Meier curves. **Results:** Generally, no significant association was found between these 2 SNPs and clinical outcomes of CRC. In stratified analysis of subgroup without adjuvant chemotherapy, patients carrying CT/TT genotypes of rs2057482 exhibited a borderline significant association with better overall survival when compared with those carrying CC genotype [Hazard ratio (HR), 0.47; 95% confidence interval (95% CI): 0.29-0.76; $P < 0.01$]. Moreover, significant protective effects on CRC outcomes conferred by adjuvant chemotherapy were exclusively observed in patients carrying CC genotype of rs2057482 and in those carrying AC/CC genotype of rs2301113. **Conclusions:** Genetic variations in *HIF1A* gene may modulate the efficacy of adjuvant chemotherapy after surgery in CRC patients.

Keywords: *HIF1A* - single nucleotide polymorphism - prognosis - colorectal cancer - adjuvant chemotherapy

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Introduction

Colorectal cancer (CRC) is the third most common malignancy with about 500,000 new cases diagnosed each year and the fourth leading cause of cancer-related death worldwide (Jemal et al., 2009; Pearson et al., 2009). Estimated 5-year survival rates range from 85% to 90% for patients with stage I disease. However, the 5-year survival rate is less than 5% for patients with stage IV disease due to the high recurrence rate (Mancuso et al., 2005; Zhang et al., 2012). Due to different tumor characteristics and patients' genetic backgrounds, the clinical outcomes of cancer patients show a great quantity of heterogeneity. Therefore, novel biomarkers associated with tumor progression is urgently needed to distinguish high-risk patients who would benefit from adjuvant therapy.

Hypoxia Inducible Factor-1 (HIF-1) is a key transcription factor that responds to changes in environmental oxygen and cell energy, which allows the cell to adapt and survive in hostile environment of normal or tumor tissues. It plays an important role in regulating angiogenesis, cell adhesion, metastatic spread and apoptosis (Semenza, 2003). HIF-1 consists of two subunits, HIF-1 α and HIF-1 β , both of which belong to the basic loop-helix Per-Aryl hydrocarbon nuclear translocator-Sim (PAS) protein family (O'Donnell et al., 2006). Hypoxic conditions allow HIF-1 α to bind to HIF-1 β and then become an active transcriptional factor, while the presence of oxygen leads to the degradation of HIF-1 α . In a previous study, the immunohistochemical analysis in 68 CRC patients has shown that high expression levels of HIF-1 α is positively associated with TNM stage, lymph

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node involvement, and distant metastasis (Wu et al., 2010). Similarly, another study has shown that HIF-1 α over-expression is associated with the poor prognosis in a cohort of 731 CRC patients (Baba et al., 2010). In addition, HIF-1 α has also been found to be associated with poor prognosis in several types of cancer, including hepatocellular carcinoma (Wada et al., 2006), gastric tumors (Zhang et al., 2013), non-small cell lung cancer (Li et al., 2013), and neuroendocrine breast cancer (Marton et al., 2012).

Single-nucleotide polymorphisms (SNPs) are attractive molecular markers for translational studies. The *HIF1A* gene is located on chromosome 14 and a number of SNPs associated with tumor development and progression have been identified in this gene. Polymorphisms C1772T (rs11549465) comprising an amino acid change from proline to serine at position 582 and G1790A (rs11549467) resulting in the substitution of alanine with threonine at position 588 have been found to be correlated with the CRC risk (Kuwai et al., 2004; Kang et al., 2011). Another study has demonstrated that there is a significant association between SNP 191T>C (rs2057482) in *HIF1A* gene and the risk of rectal cancer (Frank et al., 2010). Considering the important role of HIF-1 α in the progression of CRC, it is reasonable that polymorphisms of *HIF1A* may affect the biological behavior and prognosis of CRC. Although several studies have found that SNPs in *HIF1A* gene are associated with risk of CRC, however, few studies have investigated the association between SNPs in *HIF1A* gene and outcomes of CRC. Only one study suggested that *HIF1A* C1772T and G1790A polymorphisms were not involved in the progression or metastasis of CRC (Szkandera et al., 2010). However, many other functional SNPs in *HIF1A* gene may impact the outcomes of CRC patients in Chinese cohort. In this study, we selected 3 functional SNPs in the *HIF1A* gene and evaluated their associations with prognosis and efficacy of adjuvant chemotherapy after surgery in a Chinese cohort of 697 primary CRC patients receiving radical surgery.

Materials and Methods

Study population

Between January 2007 and June 2012, a total of 697 patients who were diagnosed as primary colorectal cancer and had complete follow-ups and clinical information were recruited. This cohort consisted of 454 patients from Department of General Surgery at Tangdu Hospital and 243 patients from Department of General Surgery at Xijing Hospital and both hospitals are affiliated with Fourth Military Medical University, Xi'an, China. All the patients who had no prior history of other cancers were newly diagnosed and confirmed by pathologists. None of the patients had been treated by surgery, chemotherapy and/or radiotherapy before enrollment into the study. All patients' demographic and clinical data were collected from medical record. There were no age, gender, and cancer stage restrictions on recruitment. 5ml of blood were extracted from all patients for genomic DNA extraction using the E.Z.N.A. Blood DNA MidiKit (Omega Bio-Tek,

Norcross, GA) in the laboratory. In this study, all analyses were restricted to Han Chinese because more than 99% of the ethnicity was Han. The Institutional Review Board at Fourth Military Medical University approved this study and all patients signed an informed consent form before enrollment.

SNP selection and genotyping

SNPs in *HIF1A* gene were selected using a set of web-based SNP selection tools (<http://snpinfo.nih.gov/snpinfo/snfunc.htm>) according to the previous description (Zhou et al., 2012). Briefly, only validated SNPs were selected, and SNPs with minor allele frequency (MAF) <5% in Han Chinese population (CHB) were excluded. Potential functional SNPs were identified to meet the following criteria: SNPs in miRNA binding sites of 3' untranslated region (UTR), SNPs in the transcription factor binding site of the 5' flanking region (2000 bp upstream from the transcript start site), SNPs in splice sites and non-synonymous SNPs in exons. If there were multiple potential functional SNPs within the same haplotype block (defined by the linkage coefficient $r^2 > 0.8$), only 1 SNP was included. We also included the SNPs which are associated with other diseases in previous studies. Finally, we identified 2 SNPs in *HIF1A* gene were selected, including 1 SNP in the 3'-UTR (rs2057482) and 1 SNP in intron region (rs2301113) which have been previously reported to be associated with disease susceptibility (Doring et al., 2010). All selected SNPs were genotyped by iPLEX genotyping system (Sequenom, San Diego, CA, USA). Genotyping was performed by laboratory personnel blinded to patients' information. Internal quality controls and negative controls were used to ensure genotyping accuracy, and 5 samples were randomly selected and genotyped in duplicate with 100% concordance.

Statistical analysis

For each SNP, additive, dominant (WV+VV vs WW) and recessive (VV vs WW+WV) genetic models were selected for analysis. Two major endpoints were evaluated in this study: overall survival (OS) and recurrence-free survival (RFS). OS was defined as the time from surgery to death from any cause. RFS was defined as the time from the date of surgery to the first date of recurrence. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated by the Cox proportional hazard model with adjusting for age, gender, smoking status, differentiation, clinical stage, histology and treatment after surgery. Kaplan-Meier curves and a log-rank test were used to assess the differences of patient groups in OS and RFS. Statistical significance was set at a level of 0.05 and all analyses were done using the SPSS software package (version 19.0, SPSS, Inc.).

Results

Distribution of patients' characteristics and prognosis analysis

In this study, a total of 697 patients with resected CRC were included (Table 1). The median age at the time of

diagnosis was 60 years (range, 15-88) for all patients, and 383 (54.9%) were males. 319 (45.8%) patients were colon cancer and 378 (54.2%) patients were rectum cancer. The pathological stages were as follows: 453 at stage I/II (65.0%) and 244 at stage III/IV (35.0%). The majority of patients (79.6%) had poor differentiated tumors, 543 (77.9%) received adjuvant chemotherapy after surgery. During the median follow-up of 29.2 months (ranging from 1.7 to 72.5), 185 (26.5%) patients died of CRC, and 208 (29.8%) patients developed recurrence. Six patients died from other causes and thus were treated as censored data in survival analysis.

Multivariate Cox regression analyses were performed to assess the prognostic effects of clinical characteristics and we found that patients with advanced TNM stage (stage III and IV) had significant higher death risk (HR=3.00, 95%CI: 2.15-4.19) and recurrence risk (HR=2.96, 95%CI: 2.15-4.07) than patients with early TNM stage.

In comparison, patients receiving chemotherapy after surgery showed a significantly decreased risk of death and recurrence (both HR were 0.54) when compared with those treated by surgery alone (Table 1). There was no association between CRC outcome and other clinical characteristics.

Association of SNPs with clinical outcome in CRC patients

Cox regression analyses were used to assess the associations of *HIF1A* SNP genotypes with CRC survival in 3 genetic models (Table 2). We found that there was no significant association between the two polymorphisms (rs2057482 and rs2301113) and OS or RFS in the multivariate analysis. Notably, our data showed that SNP rs2301113 was associated with OS or RFS of CRC patients with borderline significances in recessive model. Comparing to patients with the AC and CC genotype, those with variant alleles (CC genotype) had lower

Table 1. Distribution of Patients' Characteristics and Prognosis Analysis

Parameter	All patients, n (%) n=697	OS				RFS			
		Death, n (%) n=185	HR ^a	95% CI	p value	Recurrence, n (%) n=208	HR ^a	95% CI	p value
Gender									
Female	314 (45.1)	78 (42.2)	Ref.			84 (40.4)	Ref.		
Male	383 (54.9)	107 (57.8)	1.23	0.92-1.65	0.17	124 (59.6)	1.33	1.00-1.75	0.047
Age									
<60	358 (51.4)	94 (50.8)	Ref.			109 (52.4)	Ref.		
≥60	339 (48.6)	91 (49.2)	0.97	0.72-1.30	0.83	99 (47.6)	0.88	0.67-1.17	0.38
Hospital									
Tangdu	454 (65.1)	110 (59.5)	Ref.			117 (56.3)	Ref.		
Xijing	243 (34.9)	75 (40.5)	0.96	0.70-1.30	0.78	91 (43.8)	1.3	0.97-1.74	0.08
Locus									
Colon	319 (45.8)	84 (45.4)	Ref.			98 (47.1)	Ref.		
Rectum	378 (54.2)	101 (54.6)	1.04	0.78-1.39	0.79	110 (52.9)	0.92	0.70-1.21	0.57
Clinical stage									
I-II	453 (65.0)	88 (47.6)	Ref.			102 (49.0)	Ref.		
III-IV	244 (35.0)	97 (52.4)	3.00	2.15-4.19	<0.001	106 (51.0)	2.96	2.15-4.07	<0.001
Differentiation									
Well/ moderate	142 (20.4)	33 (17.8)	Ref.			41 (19.7)	Ref.		
Poor	555 (79.6)	152 (82.2)	1.38	0.93-2.05	0.11	167 (80.3)	1.22	0.85-1.75	0.29
Treatment after surgery									
None	154 (22.1)	43 (23.2)	Ref.			49 (23.6)	Ref.		
Chemotherapy	543 (77.9)	142 (76.8)	0.54	0.37-0.81	0.002	159 (76.4)	0.54	0.37-0.78	0.001

*Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; RFS, recurrence free survival. Significant *p* values were in bold; ^aAdjusted by gender, age, hospital, locus, clinical stage, differentiation and treatment after surgery where appropriate

Table 2. Association of HIF1A SNPs with Clinical outcome of Colon Cancer Patients

SNP	Genotypes and Genetic models	OS			RFS		
		Death/total	HR ^a (95% CI)	p value	Recurrence/total	HR ^a (95% CI)	p value
rs20574823'-UTR"	CC	125/458	Ref.		139/458	Ref.	
	CT	53/201	0.84 (0.61-1.17)	0.31	60/201	0.89 (0.65-1.20)	0.44
	TT	4/27	0.42 (0.16-1.15)	0.09	5/27	0.47 (0.19-1.16)	0.10
	Additive		0.78 (0.59-1.02)	0.07		0.81 (0.63-1.05)	0.11
	Dominant		0.79 (0.57-1.08)	0.14		0.83 (0.62-1.12)	0.22
	Recessive		0.45 (0.17-1.21)	0.11		0.49 (0.20-1.20)	0.12
rs2301113 Intron	AA	73/281	Ref.		85/281	Ref.	
	AC	89/298	1.03 (0.75-1.41)	0.86	95/298	1.00 (0.74-1.34)	0.98
	CC	22/111	0.67 (0.42-1.08)	0.10	26/111	0.68 (0.44-1.06)	0.09
	Additive		0.87 (0.71-1.07)	0.20		0.87 (0.72-1.06)	0.16
	Dominant		0.93 (0.69-1.25)	0.62		0.91 (0.69-1.20)	0.49
	Recessive		0.66 (0.42-1.04)	0.07		0.68 (0.45-1.04)	0.07

*Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; RFS, recurrence free survival. Significant *p* values were in bold; ^aAdjusted by gender, age, hospital, locus, clinical stage, differentiation and treatment after surgery where appropriate

death risk (HR=0.66, 95%CI, 0.42-1.04, $p=0.07$) and recurrence risk (HR=0.68, 95%CI, 0.45-1.04, $p=0.07$). The Kaplan-Meier curve of rs2057482 and rs2301113 were showed in Figure 1. And the log-rank tests showed no significant results for these two SNPs. We then performed a stratified analysis to evaluate OS and RFS in different strata according to CRC patients' treatment after surgery (Table 3), and found that CT/TT genotypes of rs2057482 exhibited a borderline significant association with better overall survival when compared with CC genotype in those without chemotherapy.

Association analysis of treatment protocol with outcomes in CRC patients stratified by SNPs

Although SNPs in *HIF1A* gene were not significantly associated with CRC patients' outcomes, SNPs may have

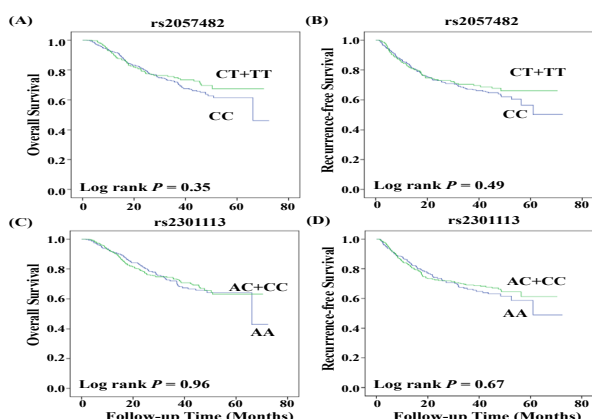


Figure 1. Kaplan-Meier Plot of rs2057482 and rs2301113 in CRC Patients. A) and B) were survival curve of rs2057482, C) and D) were survival curve of rs2301113

modifying effect on the prognostic effects of adjuvant chemotherapy in our study. To further evaluate this hypothesis, we analyzed the association between treatment protocol and outcomes in CRC patients stratified by genotypes of SNP rs2057482 and rs2301113, respectively. Surprisingly, as shown in Table 4, adjuvant chemotherapy exhibited a significant protective effect in patients with WW genotype (CC) of rs2057482 (HR=0.47 for OS, $p<0.01$; HR=0.51 for RFS, $p<0.01$) but not in patients with variant alleles (WV+VV) of rs2057482 (HR=0.73 for OS, $p=0.39$; HR=0.56 for RFS, $p=0.09$). In addition, the modulating effect of SNP rs2301113 on efficacy of adjuvant chemotherapy in CRC outcomes was observed, indicating that adjuvant chemotherapy had a significant protective effect in patients with variant alleles (AC+CC) (HR=0.56 for OS, $p=0.02$; HR=0.47 for RFS, $p<0.01$), but not in patients with wild alleles (AA) (HR=0.55 for OS, $p=0.07$; HR=0.68 for RFS, $p=0.22$).

Discussion

In the present study, we evaluated the associations between genetic polymorphisms in the *HIF1A* gene and clinical outcomes in a cohort with 697 CRC patients who received curative surgery. We do not find any statistically significant association between *HIF1A* SNPs and OS or RFS of CRC patients. However, we obtained an important finding that SNPs (rs2057482 and rs2301113) in *HIF1A* gene may modulate the protective effects of adjuvant chemotherapy on CRC patients. To the best of our knowledge, this is the first study to report that *HIF1A* gene polymorphisms may serve as an independent biomarker to predict the protective treatment response of adjuvant

Table 3. Cox Model of rs2057482 and rs2301113 in Groups of Patients with Chemotherapy or no Chemotherapy

SNP	treatment	Genotypes and best fitting model	OS			RFS		
			Death/total	HR (95%CI) ^a	p value	Recurrence/total	HR (95%CI) ^a	p value
rs2057482	No Chemotherapy	CC	30/94	Ref.		32/94	Ref.	
		CT+TT	12/57	0.54 (0.27-1.08)	0.08	16/57	0.71 (0.38-1.31)	0.27
	Chemotherapy	CC	95/364	Ref.		107/364	Ref.	
		CT+TT	45/171	0.88 (0.61-1.26)	0.48	49/171	0.88 (0.62-1.24)	0.46
rs2301113	No Chemotherapy	AA	15/59	Ref.		17/59	Ref.	
		AC+CC	27/93	0.77 (0.40-1.50)	0.44	31/93	0.89 (0.48-1.66)	0.72
	Chemotherapy	AA	58/222	Ref.		68/222	Ref.	
		AC+CC	84/316	0.96 (0.68-1.34)	0.80	90/316	0.89 (0.65-1.21)	0.45

*Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; RFS, recurrence free survival. Significant p values were in bold; ^aAdjusted by gender, age, hospital, locus, clinical stage, differentiation and treatment after surgery where appropriate

Table 4. Modulating Effects of Chemotherapy on Colorectal Cancer Survival by SNPs

SNP and variables	Death/Total	HR (95% CI) ^a	p value	Recurrence/Total	HR (95% CI) ^a	p value
By rs2057482						
In patients with WW genotype						
No Chemotherapy	30/94	Ref.		32/94	Ref.	
Chemotherapy	95/364	0.47 (0.29-0.76)	< 0.01	107/364	0.51 (0.32-0.81)	< 0.01
In patients with WV+VV genotype						
No Chemotherapy	12/57	Ref.		16/57	Ref.	
Chemotherapy	45/171	0.73 (0.36-1.50)	0.39	49/171	0.56 (0.28-1.09)	0.09
By rs2301113						
In patients with WW genotype						
No Chemotherapy	15/59	Ref.		17/59	Ref.	
Chemotherapy	58/222	0.55 (0.28-1.06)	0.07	68/222	0.68 (0.37-1.25)	0.22
In patients with WW+WV genotype						
No Chemotherapy	27/93	Ref.		31/93	Ref.	
Chemotherapy	84/316	0.56 (0.34-0.92)	0.02	90/316	0.47 (0.29-0.76)	< 0.01

**Adjusted by gender, age, hospital, locus, clinical stage, differentiation and treatment after surgery where appropriate

chemotherapy in CRC patients with radical surgery.

Several studies have performed case-control study to investigate the association between SNP rs2057482 in *HIF1A* gene and cancers. For example, a case-control study with 518 cervical cancer patients has found that a significantly increased risk of cervical cancer is associated with the CC genotype of rs2057482, and carriers of CT/TT genotypes have significantly decreased *HIF1A* mRNA expression levels compared to those with CC genotype (Fu et al., 2014). In another case-control study, they have observed the significant associations of SNP rs2057482 with risk of rectal cancer (Frank et al., 2010). In addition, another study has demonstrated that the combined variant genotypes of rs2057482 and rs11549467 are associated with increased prostate cancer risk (Li et al., 2012). However, hardly any studies have been performed to find the association between SNP rs2301113 and cancers.

A few previous studies have focused on the association between the polymorphisms including two SNPs, C1772T (rs11549465) and G1790A (rs11549467), in *HIF1A* gene and risk of CRC. For example, one study has found that there is a significant association between increased risk of developing colorectal cancer and T allele genotype of rs11549465 (Kang et al., 2011). Another study has found that rs11549465 exhibits similar results, indicating a significant association with CRC risk, but has suggested that this SNP may not be involved in progression or metastasis of CRC (Kuwai et al., 2004). In a study with a cohort of Korean patients, these two SNPs are further found not to be an independent prognostic marker for CRC patients with surgical treatment (Lee et al., 2011). Because minor allele frequencies of them are less than 5% in CHB population, we exclude these two SNPs in our study. Consistent with these reports, our study does not find any statistically significant association between two *HIF1A* SNPs and OS or RFS of CRC patients, suggesting that there may be different roles of *HIF1A* SNPs in cancer development and progression. To date, few biomarkers for prediction of adjuvant chemotherapy response in CRC have been established and proven to be valid for clinical application. In our study, we for the first time found that the patients with CC genotype of rs2057482 and CT/TT genotypes of rs2301113 can obviously benefit from adjuvant chemotherapy. Similarly, a previous study (Havelund et al., 2012) have analyzed the correlation between the rs2057482 and response to chemoradiotherapy (CRT) in rectal cancer and found positive results. However, this finding has not been replicated in validation cohort.

The mechanisms underlying the modulating role of *HIF1A* SNPs in adjuvant chemotherapy response remain to be determined. Previous studies have found that HIF-1 α overexpression indicates unfavorable prognosis and may serve as an potential prognostic factor in several cancers (Zhong et al., 1999; Marton et al., 2012; Wan et al., 2012; Li et al., 2013; Luan et al., 2013; Zhang et al., 2013;), including colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas. Furthermore, down-regulated HIF-1 α expression in transplanted esophageal squamous cell carcinoma in vivo could enhance the cytotoxicity of cisplatin (Liao et

al., 2012). One of the major mechanisms underlying the association of HIF-1 α with patients' outcomes in these cancers may be that HIF-1 α or its target genes can induces chemoresistance in many types of cancer. For example, one study has found that HIF-1 α contribute to metastasis and chemoresistance of prostate cancer and the targeted reduction of HIF-1 α expression has significantly increased the responsiveness of prostate cancer to chemotherapy (Ranasinghe et al., 2013). Also, upregulation of HIF-1 α expression appears to be a significant mechanism underlying the resistance to antiangiogenic therapies in neuroblastoma (Hartwich et al., 2013). In addition, another study has demonstrated that HIF-1 α regulates the expression of XPA and thus contributes to cisplatin resistance in lung cancer (Liu et al., 2012). The SNP rs2057482 is located in the 3' UTR region of *HIF1A* gene, which could potentially affect the microRNA binding affinity and thus influence the expression of *HIF1A*. In addition, although SNP rs2301113 is located in intron, the CHB data (<http://snpinfonihs.nih.gov/snpinfonihs/snptag.htm>) showed that rs2301113 was a tagging SNP. Many SNPs which possibly can affect the expression of HIF-1 α may have high linkage disequilibrium (LD) with rs2301113. This hypothesis needs to be clarified. These two significant SNPs in our study may change the expression of HIF-1 α and thus contribute to chemotherapy resistance in CRC patients with specific genotypes. Further functional experiments are warranted to confirm our hypothesis.

The present study had several limitations. Firstly, only 697 patients with complete clinical information and acceptable quality of genotyping assay were included for statistical analysis, so our findings need to be confirmed by additional studies with large sample size. Secondly, the exact biological significance of these SNPs needs further clarification by functional study. Moreover, our results also should be validated among different ethnic populations in the future.

In conclusion, this is the first study to suggest that polymorphisms in *HIF1A* gene may have significant impact on treatment response of adjuvant chemotherapy in Chinese CRC patients with radical surgery. Once validated, our finding would have important clinical significance for the treatment decision-making of surgical CRC patients.

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