RESEARCH ARTICLE

rs12904 Polymorphism in the 3'UTR of *EFNA1* is Associated with Colorectal Cancer Susceptibility in a Chinese Population

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Abstract

Accumulated evidence has indicated that Ephrin A1 (*EFNA1*) is associated with angiogenesis and tumorigenesis in various types of malignancies, including colorectal cancer (CRC). In the current study, we performed an online search using the public microarray database to investigate whether *EFNA1* expression might be altered in CRC tissues. We then conducted a case-control study including 306 subjects (102 cases and 204 well-matched controls) in Xiaoshan County to assess any association between genetic polymorphisms in *EFNA1* and CRC susceptibility. Searches in the Oncomine expression profiling database revealed *EFNA1* to be overexpressed in CRC tissue compared with adjacent normal tissue. The rs12904 G-A variant located in the 3' untranslated region (UTR) of *EFNA1* was observed to be associated with CRC susceptibility. Compared with the AA homozygous genotype, those carrying GA genotype had a decreased risk of developing CRC (odds ratio (OR) =0.469, 95% confidence interval (CI): 0.225-0.977, and *P* =0.043). The association was stronger among smokers and tea drinkers, however, no statistical evidence of interaction between rs12904 polymorphism and smoking or tea drinking on CRC risk was found. Our results suggest that *EFNA1* is involved in colorectal tumorigenesis, and rs12904 A>G polymorphism in the 3' UTR of *EFNA1* is associated with CRC susceptibility. Larger studies and further mechanistic investigations are warranted to confirm our findings.

Keywords: ***EFNA1*** - single-nucleotide polymorphism - colorectal cancer - susceptibility

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Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer types worldwide, with an estimation of more than 1.2 million new cases and 608,700 deaths occurred in 2008 (Jemal et al., 2011).The incidence rates of CRC are increasing rapidly in a number of countries historically at low risk, including Japan, Korea and China (Jemal et al., 2010). In Shanghai for instance, the incidence rates of CRC in both males and females increased by 50% from 1987 to 2002 (Center et al., 2009).

Environmental risk factors as well as acquired and constitutional genetic variations are considered as predominant contributors in the etiology of CRC. Modifiable risk factors include dietary factors and lifestyle-related factors such as smoking and moderate-to-heavy alcohol drinking (Huxley et al., 2009). In addition to these environmental factors, genetic variations of CRC-related genes contribute substantially to CRC risk as well. *EFNA1* maps at 1q21-q22 on chromosome 1, and encodes for Ephrin A1 which is a glycosyl-phosphatidylinositol (GPI)-anchored ligand preferentially binding to Eph receptor tyrosine kinases. *EFNA1* was originally isolated as a secreted protein in conditioned media from cultures of human umbilical vein endothelial cells treated with tumor necrosis factor-α (Holzman et al., 1990), and was found to play crucial roles in the migration and adhesion of cells during development (Poliakov et al., 2004). Recently, accumulated evidence has indicated that *EFNA1* is associated with angiogenesis and tumorigenesis in various types of malignancies. Expression of *EFNA1* was observed in various types of tumor cells and blocking *EFNA1* with soluble EphA2-Fc decreased tumor-associated angiogenesis and consequently tumor progression (Brantley et al., 2002; Dobrzanski et al., 2004). In hepatocellular carcinoma (HCC), *EFNA1* mRNA was overexpressed compared with adjacent nontumorous tissues, and the level of secreted *EFNA1* in serum samples from HCC patients were significantly higher than those from healthy controls (Cui et al., 2010). Likewise, elevated expression of *EFNA1* was observed in gastric cancer (Nakamura et al., 2005), bladder cancer (Abraham et al., 2006) and more recently, prostate cancer (Larkin et al., 2012).

Several studies investigating the role of *EFNA1* in colorectal tumorigenesis yielded similar findings. Potla

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et al. (2002) found that decreased *EFNA1* expression in HT29 colon carcinoma cells reduced the growth of the tumor cells in three-dimensional spheroid static cultures. Lips et al. (2008) and Shi et al. (2012) reported that *EFNA1* mRNA expression increased from rectal adenoma to carcinoma. Furthermore, a recent study conducted by Yamamoto et al. suggested that *EFNA1* was an independent prognostic factor for CRC, and its loss-of-function was associated with reduced proliferation and decreased invasion and migration of CRC cell lines (Yamamoto et al., 2013).

Given the apparent importance of *EFNA1* in colorectal tumorigenesis, we hypothesized that genetic variations in *EFNA1* gene may be associated with CRC susceptibility. In the current study, we first searched the public microarray database to investigate whether altered expression of *EFNA1* can be observed in CRC tissues. We then conducted a case-control study to evaluate the association between genetic polymorphisms in *EFNA1* and CRC susceptibility.

Materials and Methods

Expression analysis of EFNA1 *in CRC tissues*

We performed an online search using the Oncomine database (www.oncomine.org; last accessed on May 22, 2013) for expression array comparisons involving CRC tissues and normal controls. The key words used were: Gene "*EFNA1*"; Cancer type:"Colorectal cancer"; Analysis type: "Cancer vs. Normal Analysis"; and the results were filtered by $P \leq 0.005$ and fold change $\geq |2|$. The search returned two arrays: the "Notterman Colon" which compares *EFNA1* expression in colon adenocarcinoma and paired adjacent normal tissues, and the "Kaiser Colon" which compares *EFNA1* expression in colorectal adenocarcinoma and normal colorectal tissues. Detailed information regarding the tissue sample collection and experimental protocols can be found in the Oncomine database or from the original publications (Notterman et al., 2001; Kaiser et al., 2007).

Study subjects

CRC patients were recruited from four local hospitals from May 2005 to October 2009. Eligible cases were incident and histologically confirmed CRC, living in Xiaoshan County for ≥ 20 years, mentally competent to complete the interview and with no previous diagnosis of familial adenomatous polyposis, ulcerative colitis or Crohn's disease. Healthy controls with no previous history of cancer were recruited in parallel and 1:2 matched to cases by age (± 5 years), gender, ethnicity, and residential area. Face-to-face interviews were conducted by trained interviewers, who administered a structured questionnaire relating to demographic characteristics (e.g., age, gender, body mass index (BMI), marital status, occupation and education level), family history of cancer, menopausal history, and lifestyle-related factors (e.g., diet, smoking, alcohol drinking and tea drinking). After interview, 2ml blood sample was collected into sodium citrate anticoagulant tubes and stored at -80°C for DNA isolation. A total of 102 cases and 204 well-matched controls with DNA samples available were included in the current study. All the study procedures were reviewed and approved by the local medical ethical committee and written informed consents were obtained from all study subjects before participation.

SNP selection and Genotyping

Genomic DNA was isolated from peripheral blood samples for each study subject using the modified salting-out procedure (Nasiri et al., 2005). Single nucleotide polymorphisms (SNPs) in the genomic region from 1,500bp upstream to 1,500bp downstream of *EFNA1* with minor allele frequencies (MAF) of > 0.1 within the Han Chinese in Beijing (CHB) populations were identified from the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Tag SNPs representing SNPs with pairwise correlation of $r^2 > 0.8$ were selected using the Tagger algorithm implemented in the Haploview interface (Barrett, 2009). rs12904 which captures 100% alleles (Tag SNPs rs4971066, rs9297, rs4745 and rs12904) in *EFNA1* with mean r^2 of 0.978 was chosen. Genotyping was performed using the Sequenom MassArray genotyping platform (Sequenom, Inc., San Diego, CA, USA). Blinded replicate samples (10% of the samples) and negative controls (one for each 96-well plate) were interspersed throughout the genotyping assays. The call rate for the SNP genotyped was >99%, and the concordance rate for these quality control samples was 100%.

Statistical analyses

All statistical analyses were performed using the SAS statistical software, version 9.2 (SAS Institute, Cary, NC, USA), unless otherwise noted. Differences in the distribution of demographic and lifestyle characteristics between cases and controls were assessed using t-test for continuous variables and Pearson χ^2-test for categorical variables. Departures from Hardy-Weinberg equilibrium (HWE) were tested among controls using goodness-of-fit χ^2 analysis. Associations between rs12904 polymorphism and CRC risk were assessed by conditional multivariate logistic regression models including the following covariates: age, education level, occupation, cigarette smoking, alcohol drinking and tea drinking. Stratified analysis was also performed to explore potential gene-environment interactions and odds ratios (ORs) and 95% confidence intervals (CIs) were determined using unconditional logistic regression. Two-sided P-values of less than 0.05 were considered as statistically significant.

Results

We searched the Oncomine expression profiling database to determine whether *EFNA1* expression was altered in patients with CRC relative to controls. We used the keywords "*EFNA1*" and "colorectal cancer" and conditional filters of $P \leq 0.005$ and fold change $\geq |2|$. The search returned two arrays: the "Notterman Colon" and the "Kaiser Colon". The study of "Notterman Colon" examined gene expression in colon adenocarcinoma and paired adjacent normal tissues. As shown in Figure 1A, *EFNA1* expression was increased in 14 out of 18

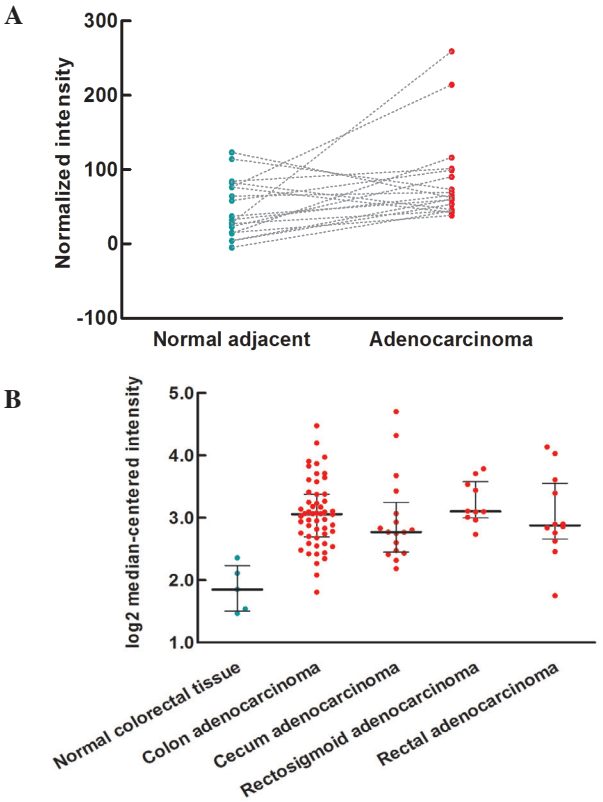

Figure 1. *EFNA1* Gene Expression Analysis in CRC Tissues from the Oncomine Expression Profiling Database. A. The "Notterman Colon" examined gene expression in colon adenocarcinoma and paired adjacent normal tissue. *EFNA1* expression was increased in 14 out of the18 adenocarcinoma-adjacent normal tissue pairs. B. In the study of "Kaiser Colon" which examined gene expression in colon, cecum, rectal-sigmoid and rectal adenocarcinoma and normal colon tissues, *EFNA1* expression was significantly lower in normal colorectal tissues than in adenocarcinoma tissues (P =8.64×10^{-5} - 0.001, fold change = 1.780-3.486). The median expression level of *EFNA1* and its interquartile range were shown for each subgroup

adenocarcinoma-adjacent normal tissue pairs ($P < 0.005$, fold change = 2.588). Likewise, in the study of "Kaiser Colon" which examined gene expression in colon, cecum, rectal-sigmoid, rectal adenocarcinoma and normal colorectal tissues, *EFNA1* expression was significantly lower in normal tissues than in adenocarcinoma tissues (P = 8.64×10^{-5} - 0.001, fold change = 1.780-3.486) (Figure 1B). However, no statistically significant associations between tumor site, Dukes stage, tumor size and *EFNA1* expression were found.

Table 1 summarizes the distributions of demographic characteristics and selected risk factors for the CRC case-control study. Because of 1:2 matching used in the study design, no significant differences in the distributions of age, gender, ethnicity and residential area between cases and controls were observed. CRC cases were more likely to be farmers compared with controls ($P = 0.013$), but no other demographic characteristics and lifestyle-related factors such as smoking, alcohol drinking and tea drinking status differed significantly between cases and controls. The tag SNP rs12904 located in the 3' untranslated region (UTR) of *EFNA1* was chosen to represent all the variation in this gene region. No significant departure from Hardy-Weinberg equilibrium was observed among the controls (P =0.677). The genotype distributions between cases and controls are listed in Table 2. In the unstratified analysis, the association between variant allele of rs12904 and CRC risk was found to be statistically significant. Compared with rs12904 AA homozygous genotype, those carrying GA genotype had a decreased risk of developing CRC (OR=0.469, 95% CI: 0.225-0.977). However, under the dominant model, rs12904 A>G was borderline associated

Table 1. Selected Characteristics of the Study Population

	Cases (n = 102)		Controls (n = 204)		
Variables	N	%	N	%	*P*-value[a]
Age (years)					
< 65	50	49.0	120	58.8	0.104
≥65	52	51.0	84	41.2	
Sex					
Males	44	43.1	88	43.1	1.000
Females	58	56.9	116	56.9	
Educational level					
Illiterate	54	52.9	81	39.7	0.090
Primary school	42	41.2	104	51.0	
Middle school and above	6	5.9	18	8.8	
Marital status					
Married	97	95.1	190	93.1	0.834
Others	5	4.9	11	5.4	
Occupatin					
Farmer	73	71.6	118	57.8	0.013
Others	27	26.5	84	41.2	
Cigarette smoking					
No	61	59.8	117	57.4	0.682
Yes	41	40.2	87	42.6	
Alcohol drinking					
No	57	55.9	119	58.3	0.683
Yes	45	44.1	83	40.7	
Tea drinking					
No	53	52.0	118	57.8	0.306
Yes	49	48.0	85	41.7	

*Subjects with missing information were not included in the

Table 2. Association Between *EFNA1* Polymorphism and Colorectal Cancer Risk*

Polymorphism	Controls, N (%)	Cases, N (%)	OR (95% CI)	*P*-value	Adjusted OR (95% CI)[a]	Adjusted *P*-value[a]
EFNA1 A>G rs12904						
AA	121 (59.6)	69 (69.0)	Reference		Reference	
GA	73 (36.0)	27 (27.0)	0.605 (0.351-1.043)	0.071	0.469 (0.225-0.977)	0.043
GG	9 (4.4)	4 (4.0)	1.000 (0.277-3.608)	1.000	1.293 (0.254-6.594)	0.757
Dominant Model	GA+GG vs. AA		0.673 (0.403-1.123)	0.130	0.557 (0.290-1.070)	0.079
Recessive Model	GA+AA vs. GG		0.889 (0.274-2.886)	0.845	1.423 (0.274-7.398)	0.675

*Subjects with missing information were not included in the analyses; [a]Adjusted for age, education level, occupation, smoke inhalation, alochol drinking and tea drinking

Table 3. Stratification Analyses of *EFNA1* Polymorphism with Colorectal Cancer Risk*

Variables	Controls, N (%)	Cases, N (%)	OR (95% CI)	*P*-value	Adjusted OR (95% CI)[a]	Adjusted *P*-value[a]
Cigarette smoking						
Yes						
rs12904						
AA	54 (62.1)	31 (79.49)	Reference		Reference	
GA	28 (32.2)	8 (20.51)	0.400 (0.142-1.131)	0.084	0.100 (0.014-0.715)	0.022
GA+GG	33 (37.9)	8 (20.51)	0.355 (0.128-0.986)	0.047	0.098 (0.014-0.685)	0.019
No						
rs12904						
AA	67 (57.76)	38 (62.30)	Reference		Reference	
GA	45 (38.79)	19 (31.15)	0.862 (0.404-1.840)	0.701	0.761 (0.287-2.020)	0.584
GA+GG	49 (42.24)	23 (37.70)	1.063 (0.536-2.111)	0.861	0.919 (0.409-2.065)	0.838
Alochol drinking						
Yes						
rs12904						
AA	49 (59.04)	28 (65.12)	Reference		Reference	
GA	30 (36.14)	13 (30.23)	0.588 (0.226-1.529)	0.276	0.492 (0.122-1.977)	0.317
GA+GG	34 (40.96)	15 (34.88)	0.592 (0.243-1.441)	0.248	0.690 (0.198-2.406)	0.560
No						
rs12904						
AA	71 (60.17)	41 (71.93)	Reference		Reference	
GA	42 (35.59)	14 (24.56)	0.785 (0.320-1.926)	0.597	0.599 (0.184-1.954)	0.396
GA+GG	47 (39.93)	16 (28.07)	0.864 (0.380-1.967)	0.728	0.687 (0.258-1.826)	0.452
Tea drinking						
Yes						
rs12904						
AA	46 (54.12)	34(72.34)	Reference		Reference	
GA	34 (40.00)	12(25.53)	0.149 (0.033-0.672)	0.013	0.116 (0.012-1.094)	0.060
GA+GG	39 (45.88)	46(27.66)	0.128 (0.028-0.576)	0.007	0.106 (0.012-0.975)	0.048
No						
rs12904						
AA	74 (63.25)	35 (66.04)	Reference		Reference	
GA	39 (33.33)	15 (28.30)	1.227 (0.508-2.966)	0.650	0.812 (0.222-2.965)	0.752
GA+GG	43 (46.75)	65 (33.96)	1.293 (0.577-2.897)	0.533	0.867 (0.315-2.387)	0.783

*Subjects with missing information were not included in the analyses; [a]Adjusted for age, education level, occupation, smoke inhalation, alochol drinking and tea drinking

with a decreased CRC risk (OR=0.557, 95% CI: 0.290-1.070).

In the following stratified analyses by lifestyle-related factors such as smoking, alcohol drinking and tea drinking, only in the subgroups of smokers (GA vs. AA: OR=0.100, 95% CI: 0.014-0.715; GA+GG vs. AA: OR=0.098, 95% CI: 0.014-0.685) and tea drinkers (GA+GG vs. AA: OR=0.106, 95% CI: 0.012-0.975) did the decreased risk remain statistically significant (Table 3), though the subgroups had limited observations. No statistical evidence of interaction was found between rs12904 polymorphism and smoking or tea drinking on CRC risk.

Discussion

In the present study, we examined *EFNA1* expression in CRC tissues using the public microarray database and assessed the genetic association between *EFNA1* polymorphisms and CRC susceptibility. We found elevated *EFNA1* expression in colorectal adenocarcima in comparison to normal controls, suggesting *EFNA1*'s involvement in colorectal tumorigenesis, and its potential role as a diagnostic biomarker in CRC characterization. In our case-control study, we found that rs12904 G/A variant was significantly associated with a decreased risk of developing CRC compared with AA genotype. In the following stratified analyses, such an effect was more evident in the subgroups of smokers and tea drinkers, which could be chance findings because of the limited observations in each subgroup. Interestingly, a recent case-control study conducted by Li et al. reported that *EFNA1* rs12904 was associated with gastric cancer risk in a Chinese population (Li et al., 2012), which is in agreement with our finding that rs12904 G variant allele is protective against cancer susceptibility.

rs12904 resides in the 3'UTR of *EFNA1* gene, which suggests that it may have the potential to interfere with *EFNA1* mRNA stability and translation through altering miRNA:mRNA interactions. Although the role of miRNAs in CRC pathogenesis has been established by the identification of miRNA expression signatures that characterize normal and tumor phenotypes as well as numerous oncogenes and tumor suppressors as miRNA targets, few studies have investigated the role of SNPs in the miRNA binding sites in the etiology of CRC (Yang et al., 2009; Landi et al., 2012). The majority of miRNAs bind to target sequences located in the 3'UTR of mRNAs by base pairing, resulting in the cleavage of target mRNA or repression of their translation. When SNPs occur in the 3'UTR, they may modulate gene expression by altering miRNA target binding capacity, ultimately leading to

differences in the disease susceptibility (Nicoloso et al., 2010). As predicted by TargetScan (http://www.targetscan.org) and miRdSNP (http://mirdsnp.ccr.buffalo.edu), rs12904 A>G polymorphism may have the potential to affect the binding of three miR-200 family members miR-200c, miR-429 and miR-200b, which have been shown to play key roles in epithelial-mesenchymal transition (EMT) (Burk et al., 2008). miR-200c, miR-429 and miR-200b were highly expressed in colorectal cell lines, and altered expression of miR-200c and miR-429 were observed in CRC tissues, and moreover, high level of miR-200c was associated with poor prognosis in CRC (Cummins et al., 2006). However, only one study examined the function of rs12904 on miR-200c binding capacity in gastric cancer cell lines to date, which showed that rs12904 G>A change resulted in altered regulation of miR-200c on luciferase expression, and *EFNA1* expression was significantly higher for rs12904 AA genotype than for AG or GG genotype (Li et al., 2012). Further studies are needed to elucidate the exact functional impact of *EFNA1* rs12904 polymorphism and its interaction with miRNAs especially miR-200 family in colorectal tumorigenesis.

Although the present study, to our knowledge, is the first study on *EFNA1* polymorphisms and CRC risk, our findings are best considered preliminary. Because of the limited sample size, the statistical power may not be adequate to detect weak gene-disease associations and gene-environment interactions. Moreover, the lack of supporting experimental information on the functional nature of rs12904 polymorphism precluded the possibility of corroborating the genetic association results with the findings from other analyses of our study. Therefore, larger studies are warranted to further assess the association between rs12904 and CRC risk and the exact function of the SNP in the etiology of CRC.

In summary, our results suggest that *EFNA1* is involved in colorectal tumorigenesis, and rs12904 A>G polymorphism in the 3' UTR of *EFNA1* is associated with decreased CRC susceptibility. Larger studies and further mechanistic investigations are warranted to confirm our findings.

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