

# Single nucleotide polymorphism rs4648298 in miRNAs hsa-miR21 and hsa-miR590 binding site of COX gene is a strong colorectal cancer determinant

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**Background:** Genetic determinants are considered as driving forces in development colorectal cancer (CRC), a malignancy that ranks as the second cause of cancer death in the world. Single nucleotide polymorphisms (SNPs), are considered as the main genetic factor in cancers susceptibility. MicroRNAs are critical players in posttranslational gene regulation by binding to their specific recognition sequences located at 3' untranslated region (UTR) of mRNAs. In present study we have elucidated the role of 9,850 A>G (rs4648298), in development of sporadic CRC in Iranian population.

**Methods:** A case-control study using 88 CRC patients and 88 noncancerous counterparts was undertaken in order to determine rs4648298 genotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Also, a meta-analysis was performed based on 9 articles accessed via the MEDLINE, Cochrane review, Google Scholar and Scopus databases.

**Results:** AA genotype was determined to be associated with significant decreased risk of CRC in our study population [odds ratio (OR) =0.14; 95% confidence interval (CI), 0.05–0.34; P<0.001]. In a meta-analysis on 6 risk estimates for the AG versus AA genotype, we found a significant inverse association between AG SNPs and risk of gastric adenocarcinoma, CRC, breast cancer and prostate cancer (OR =0.86; 95% CI, 0.76–0.98; P<0.02).

**Conclusions:** Our results suggest significant correlation between rs4648298 polymorphism and CRC risk in Iranian population.

**Keywords:** COX gene; microRNA; single-nucleotide polymorphism; colorectal cancer (CRC)

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## Introduction

Colorectal cancer (CRC) rated as the third diagnosed malignancy and fourth most common cause of cancer mortality worldwide. In developed regions, the occurrence of CRC for men and women increases to 37.7 and 24.3 per

100,000 persons per year (1,2). However the incidence of CRC is lower in Asian than in western countries, recent studies have shown, increasing rates of CRC in Asia and especially in Iran as a developing country (3,4). Although previous reports revealed that many environmental and

lifestyle factors such as high body mass index (BMI), smoking, low activity and alcohol drinking increase the risk of CRC (5,6). Albeit, from all people who are exposed to same risk factors just some of them developed CRC. It could interpret that CRC is a complex disease, influenced by genetics and environmental factors. MicroRNAs constitute a novel discovered class of small non-coding RNAs (about 22 nucleotides) that play important roles in the regulation of gene expression by binding to the 3'-untranslated regions (3'-UTR) of specific mRNAs, and leading to mRNA cleavage or translational repression. Accumulated evidences demonstrated that miRNAs are one of the key players in cell differentiation, growth, apoptosis and insulin secretion (7,8). The most frequent type of variations in the human genome is single nucleotide polymorphism (SNP) that they exist once almost in every 300 nucleotides. Several studies demonstrated that SNPs in 3'UTR of mRNA may impact microRNAs functions by influence in secondary structure of 3'UTR and thermodynamic features of hybridization site (7,9,10). These SNPs can deregulate expression of target gene by change in binding capacity of miRNAs. The association between inflammation and tumorigenesis especially in CRC is well-established. COXs are rate-limiting enzymes that mediate the formation of prostaglandins from arachidonic acid (11,12). Two major cyclooxygenase isoforms are Cyclooxygenase-1 (COX-1) and COX-2 with 60% homology, most tissues express COX-1 at relatively low levels (13). Cyclooxygenase-2 (COX-2) is the inducible isoform that is not found in most normal tissues but can be induced by cytokines (inflammatory reactions), growth factors or tumor promoters (14-17). In overall COX-2 could be known as an acute response protein that is expressed as part of respond to inflammation (18). Overexpression of COX-2 is sufficient for carcinogenesis in animal models and blockage of the COX-2 pathway results in decrease in tumor occurrence and progression (19). COX-2 overexpression is observed in multiple cancers especially sporadic CRC (20,21). Several studies indicate that SNPs in the COX-2 gene may modulate the risk of CRC development (12,14,22). As mentioned polymorphisms in 3'UTR of COX-2 gene may affect the expression by modifying of miRNAs binding capacity. In this case control study we assessed association between SNP rs4648298 in 3'UTR of COX2 gene with sporadic CRC risk in Iranian population. Investigate on miRNA and SNP databases (such as miRBase, miRanda and mirdsnp) shows that this polymorphism (rs4648298) is in miRNA binding site of hsa-miR21, hsa-miR590 and in vicinity of

has-miR101 binding site that could influence on miRNA-mRNA interaction. Non-steroidal anti-inflammatory drugs (NSAIDs) known as reducer of CRC risk due to their significant roles in decreasing of inflammation, especially by inhibition of COX-2 expression; therefore in this study we also assessed interaction between SNP rs4648298 and use of NSAIDs in our case—control study. Moreover, we compared our results with previously published results for rs4648298 using meta-analysis.

## Methods

### Study population

For this case control study, during the 2-year period from mid-2013 to mid-2015, totally 176 participants were selected amongst patients referred to the colonoscopy centers of the University Hospitals, Isfahan, Iran. CRC cases, 88, were first diagnosed by colonoscopy and then followed their pathology report for the ultimate confirmation of their colonoscopy based CRC diagnosis. Controls, 88, were also selected from the same population with no signs of CRC detected in colonoscopy examination. The study was approved by Isfahan University of Medical Sciences Ethics board (approval number 392263) and written informed consent was filled and signed by all participants. To eliminate any established genetic risk factor for CRC, patients were individually interviewed to select cases with sporadic CRC without positive history of familial cancers. Also all participants were asked to fill up a questionnaire in order to register the parameters known to influence the CRC susceptibility risk including gender, age, BMI, physical activity, smoking status and NSAIDs consumption.

### Genotyping of polymorphism

Approximately, 5 mL venous blood was collected from each participant. DNA was extracted by Prime Prep Genomic DNA Isolation Kit (GeNet Bio). The quality and quantity of the extracted DNA was assessed by agarose gel electrophoresis and spectroscopy, and then DNA was stored at  $-20^{\circ}\text{C}$  until analyzed. Polymorphism of COX-2 gene was genotyped by PCR-RFLP method. Using appropriate primer set, 392 bp fragment of COX-2 gene that contained the selected polymorphism was amplified by PCR. Thermal cycling contained an initial denaturation of  $94^{\circ}\text{C}$  for 4 min and then 33 cycles including  $94^{\circ}\text{C}$  for

**Table 1** Primer sequence and characteristic of PCR product and digestion condition

SNP ID	Primer sequence	PCR product length (bp)	Restriction enzyme (Ta °C)	RFLP fragment size (bp)
rs4648298	F: TTCCACATCTCATTGTCACTG; R: AATTAACGAAGCATCCACAG	392	AluI (37 °C)	AA: 392; GG: 294+98; AG: 98+294+392

PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism.

30 s, 53 °C for 30 s, and 72 °C for 30 s after these cycles final extension of 72 °C for 5 min. Restriction fragment length polymorphism (RFLP) was carried out using the AluI restriction endonuclease. PCR product size, restriction enzyme used, fragments subsequent to digestion and primer sequences used, are given in the *Table 1*. Finally the result of genotyping was confirmed by randomly selected samples for direct sequencing.

### Meta-analysis

#### Methods and search strategy

We had intended to summarize data on relationship between SNP rs4648298 in 3'UTR of *COX-2* gene and cancer risk.

In this field, we conducted a literature search, up to Jul 2017, of MEDLINE, Cochrane review, Google Scholar and Scopus databases for related studies. The search terms were “Neoplasms” (MeSH) AND “SNPs” (MeSH) AND “rs4648298” (tiab) AND “COX-2” (tiab).

Studies were eligible for inclusion in the current analysis if: (I) SNP rs4648298 in 3'UTR of *COX-2* gene was assessed; (II) their final outcome was cancer risk; and (III) estimates of relative risks (RR), hazard ratios (HR) or odds ratio (OR) with corresponding 95% confidence interval (CI) for the cancer risk among SNP genotypes were provided. Two investigators extracted data independently, and any discrepancies were resolved by discussion.

#### Statistical analysis

Odds ratio were used as the measure association between SNP rs4648298 in 3'UTR of *COX-2* gene and cancer risk. Meta-analyses were performed using the random-effects model that was presented as forest plots with 95% CI. The statistical heterogeneity of the studies was calculated with the Cochran's Q and I<sup>2</sup> index (I<sup>2</sup>>50% was considered as significant heterogeneity). To assess the impact of possible factors on pooled effect size and heterogeneity, subgroup analyses were performed. Publication bias was assessed using the funnel plot method and Egger's test. Statistical

analyses were conducted using the statistical software package Stata version 11.2. P values less than 0.05 were considered statistically significant.

### Statistics

Genotype frequencies in case and controls were tested for Hardy-Weinberg equilibrium using  $\chi^2$  test. Associations between COX-2 (rs4648298) polymorphism with susceptibility to sporadic CRC were examined by logistic regression analysis. Correlation among this polymorphism and CRC was evaluated using ORs and 95% CIs. The significance level was set at P<0.05. The difference in demographic and lifestyle characteristics distribution such as age, gender, smoking status, BMI and NSAIDs consumption, assessed by Pearson  $\chi^2$  test for categorical variables and *t*-test for continuous variables. Mann-Whitney test were used to compare physical activity between CRC and control groups. All statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA).

## Results

### Demographic and lifestyle characteristics

To evaluate correlation between risk factors (genetic and environmental) with CRC incidence, we analyzed 176 total participants in case and control groups; 88 patients (40 males and 48 females) in case and 88 (45 males and 43 females) healthy subjects in control group. There was no significant association between case and control group regarding age (P=0.56) and gender (P=0.45), this illustrating that for these variables matching was adequate. Also for smoking status (P=0.50) and mean BMI (P=0.33) we could not found any difference among two groups. However we found significant difference between control and case group in term of physical activity (OR =0.58, 95% CI, 0.41–0.83). Also this study demonstrated that individuals in control group were more NSAIDs consumer compared to patient group (OR =0.30, 95% CI, 0.13–0.68). Characteristics of study population are summarized in *Table 2*.

**Table 2** Baseline characteristics of CRC patients and control subjects participated in study

Characteristics	Controls (N: 88)	Cases (N: 88)	P value
Age (mean ± SD)	58.34±10.412	57.26±11.569	0.56
Gender			0.45
Male	45 (47.7%)	40 (52.3%)	
Female	43 (52.3%)	48 (47.7%)	
Smoking			0.50
Yes	15 (19.3%)	15 (17%)	
No	73 (80.7%)	73 (83%)	
NSAIDs			0.002*
Irregular	62 (67%)	78 (86.4%)	
Regular	26 (33%)	10 (13.6%)	
Physical activity			<0.001*
Very low	4 (4.5%)	24 (27.3%)	
Low	38 (43.2%)	33 (37.5%)	
Moderate	34 (38.6%)	24 (27.3%)	
High	12 (13.6%)	7 (8.0%)	
BMI (mean ± SD) kg/m <sup>2</sup>	25.08±3.89	25.68±4.37	0.33

\*, P<0.05; CRC, colorectal cancer; NSAIDs, non-steroidal anti-inflammatory drugs.

### Genotype and allele distribution

The genotype distribution of rs4648298 polymorphism in two groups was in Hardy-Weinberg equilibrium. Genotype distribution in current study indicated that homozygous variant (GG) was not found in case and control group. The frequency of AA genotype in case and control group was 64.8% and 93.2% respectively and frequency of AG genotype in case and control group was 35.2% and 6.8% respectively (Table 3). In this evaluation we found significant association between AA genotype and decrease risk of CRC (OR =0.14; 95% CI, 0.05–0.34; P<0.001). Also in allele distribution we found that A allele have high frequency in control group compared to case group and we found that A allele was associated with decrease risk of CRC (OR= 0.17; 95% CI, 0.07–0.04; P<0.001). Furthermore, we assessed association between COX-2 polymorphism, rs4648298, genotypes and sporadic CRC risk in subgroups of participants that categorized by age (<55 and ≥55), gender

(male and female), smoking status (smoker and nonsmoker) and NSAIDs consuming (regular and irregular) (Table 4). Data indicated that there was significant difference in each sub group of age, NSAIDs consuming and gender between case and control group regarding to CRC risk. This means that in these subgroups, AA genotype have protective effect for CRC similar to overall. However in smoker subgroup there was no association between this polymorphism and CRC risk (P=0.17), but in nonsmoker subgroup AA genotype was correlated with decrease risk of CRC (P<0.001).

### Findings from meta-analysis

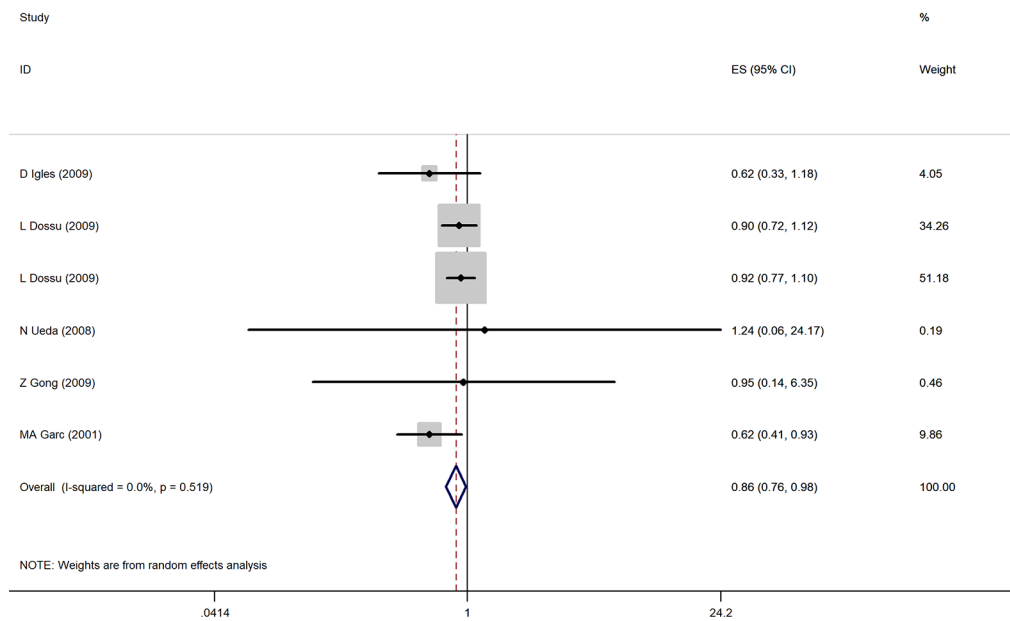
We identified nine studies including gastric adenocarcinoma (23), CRC (14,15,24-26), breast cancer and prostate cancer (27) and lymphoma (28) that met the inclusion criteria of this meta-analysis. The cancer risk for AG + GG and AG genotypes compared to AA SNPs was assessed.

In a meta-analysis on six risk estimates for the AG versus AA genotype, we found a significant inverse association between AG SNPs and risk of gastric adenocarcinoma, CRC, breast cancer and prostate cancer (OR =0.86; 95% CI, 0.76–0.98; P<0.02; Figure 1). Publication bias was not observed by Egger's test (P=0.49). Also, there were not asymmetries in funnel's plots in the current analyses. Also, a between-study heterogeneity was not found (I<sup>2</sup>=0.0%, P=0.519).

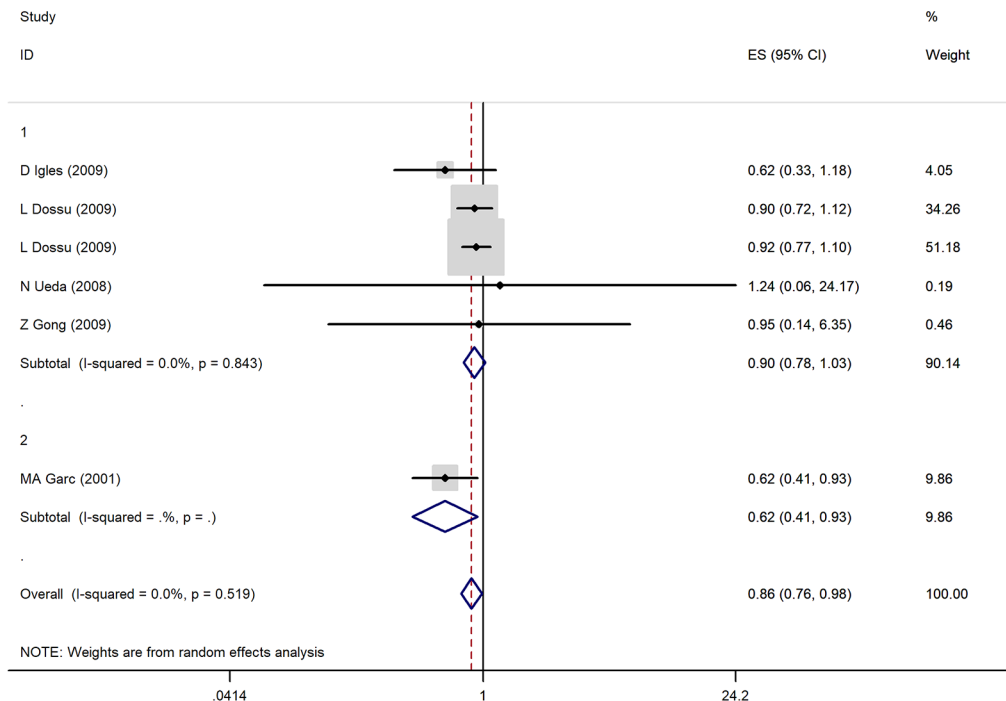
Through subgroup analysis by studies design, among case-control studies (ID =1), we found that a non-significant protective effects in AG compared to AA polymorphism in cancers risk (OR =0.90; 95% CI, 0.87–1.03; P=0.11). However, there was a significant association between AG genotype and gastric adenocarcinoma in the MA Garc study with cohort design (OR =0.62; 95% CI, 0.41–0.93; P=0.02; Figure 2). Furthermore, individuals with AG + GG variant in rs4648298 in 3'UTR of COX-2 gene compared with AA had not a significant less odd for CRC, breast cancer and lymphoma among four studies (OR =0.87; 95% CI, 0.54–1.41; P<0.57; Figure 3). The test of between-studies heterogeneity was not significant (I<sup>2</sup>=41.8%, P=0.161). Also, funnel plots and Egger's test (P=0.40) indicated no publication bias in studies.

### Discussion

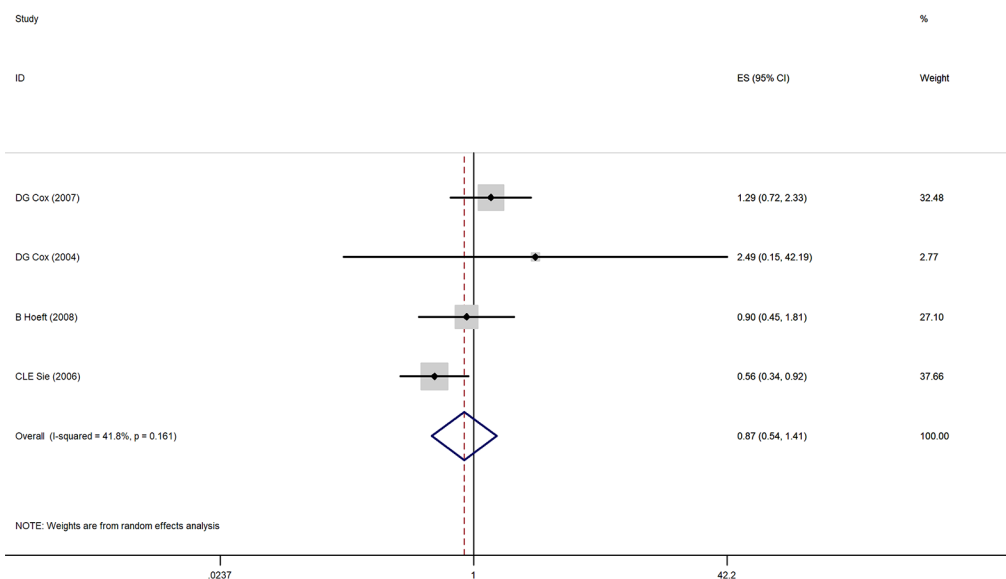
To the best of our knowledge present study is the



**Figure 1** Odds ratio of cancer among individuals with AG SNPs compared those with AA genotype. SNPs, single nucleotide polymorphisms.



**Figure 2** Odds ratio of cancer among individuals with AG SNPs compared those with AA genotype by study design type subgroup. SNPs, single nucleotide polymorphisms.



**Figure 3** Odds ratio of cancer among individuals with AG + GG SNPs compared with those with AA genotype. SNPs, single nucleotide polymorphisms.

**Table 3** Association between genotypes and allele frequency with CRC risk

Genotype subgroups	Case no. (%)	Control no. (%)	P value	OR (95% CI)
<b>Genotype frequency</b>				
AA	57 (64.8)	82 (93.2)	<0.001*	0.14 (0.05–0.34)
AG	31 (35.2)	6 (6.8)		
<b>Allele frequency</b>				
A	145 (82.4)	170 (96.6)	<0.001*	0.16 (0.06–0.4)
G	31 (17.6)	6 (3.4)		

\*, P<0.05; CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.

first report in Iranian population that investigates the correlation between COX-2 polymorphism, rs4648298, with CRC risk. COX-2 or PTGS2 enzyme as an inducible isoform of cyclooxygenase is involved in prostaglandin synthesis from arachidonic acid. Induction of COX-2 by growth factors, inflammatory cytokines and tumor promoters lead to production of prostaglandins that could stimulate the inflammation, cell proliferation, angiogenesis and differentiation (29,30). Previous study demonstrated that COX-2 is overexpressed in both sporadic colorectal adenomas and carcinomas (31). On the other hand a growing body of evidence proposed that NSAIDs are associated with decrease risk of CRC by inhibition of COX-2 (32-34).

MicroRNAs (miRNAs) are small non-coding RNAs involved in posttranslational gene expression regulation. Base pairing with specific sequence at 3'UTR of target mRNAs is key feature for miRNAs to execute this regulatory function (7,8). SNPs located in miRNA binding site contribute to the disruption of miRNA recognition elements (MREs) or create new sites resulting in up- or down-regulation of target genes (35,36). In addition previous studies revealed that in 3'UTR of COX-2 gene there were AU-rich elements which are binding sites for proteins and inflammatory mediators that could modulate stability of COX-2 mRNA thus SNPs in this region could disturb these interactions (37,38).

In current study we evaluate association between a

**Table 4** Stratification analyzes of the COX-2 polymorphism (rs4648298) in patients and controls

Group	Genotype		P value	OR (95% CI)
	AA (%)	AG (%)		
Overall				
Case	57 (64.8)	31 (35.2)	<0.001*	0.14 (0.05–0.34)
Control	82 (93.2)	6 (6.8)		
Age <55				
Case	22 (37.9)	14 (87.5)	<0.001*	0.08 (0.01–0.42)
Control	36 (62.1)	2 (12.5)		
Age ≥55				
Case	35 (43.2)	17 (81.0)	0.001*	0.17 (0.05–0.57)
Control	46 (56.8)	4 (19.0)		
Irregular NSAIDs consuming				
Case	52 (46.4)	26 (92.9)	<0.001*	0.06 (0.01–0.29)
Control	60 (53.6)	2 (7.1)		
Regular NSAIDs consuming				
Case	5 (18.5)	5 (55.6)	0.015*	0.18 (0.03–0.93)
Control	22 (81.5)	4 (44.4)		
Smoker				
Case	11 (45.8)	4 (66.7)	0.17	0.42 (0.06–2.76)
Control	13 (54.2)	2 (33.3)		
Non smoker				
Case	46 (40.0)	27 (87.1)	<0.001*	0.09 (0.03–0.30)
Control	69 (60.0)	4 (12.9)		
Male				
Case	27 (39.1)	13 (81.3)	0.001*	0.14 (0.03–0.57)
Control	42 (60.9)	3 (18.8)		
Female				
Case	30 (42.9)	18 (85.7)	<0.001*	0.12 (0.034–0.464)
Control	40 (57.1)	3 (14.3)		

\*, P&lt;0.05; NSAIDs, non-steroidal anti-inflammatory drugs; OR, odds ratio; CI, confidence interval.

COX-2 polymorphism (rs4648298) and CRC risk in Iranian population. This polymorphism is located in the recognition position of miR-590, miR-21 and vicinity of miR-101 binding sites at 3'UTR of *COX-2* gene thus could affect on binding capacity of this miRNAs. Our results demonstrated that AA genotype and A allele were more frequent in control group compared with AG genotype

and G allele. It could be interpreted that AA genotype and A allele have protective effect for healthy subjects ( $P_{AA \text{ and } A} < 0.001$ ).

Also the results obtained from the meta-analysis showed a significant inverse association between AG SNPs and risk of all cancers. Several studies carried out on association between this polymorphism and risk

of different cancer types. Two reports in Brazilian and Caucasian populations demonstrated that there was no association between this polymorphism and breast cancer risk (39,40), also in a study in North Carolina (USA) on colorectal adenoma no association was found (15). In Spanish population G allele was reported to be associated with longer survival and AG genotype was associated with clinicopathological features and rs4648298 (AG genotype) known as a good prognostic indicator for CRC patients (14). While Cox *et al.* in same population indicated that individuals with AG + GG genotypes have higher risk of CRC (24). Also in Caucasians AA genotype was correlated with advance stage and decrease of overall survival in patient with gastric adenocarcinoma (23). Therefore based on our results protective effect for AA genotype and A allele in healthy subjects was not in agreement with previous studies. The reasons for the contradictory results may be due to differences in ethnic background and different types of cancers. However, performing replicative studies in every population is necessary to validate these results. Stratification of participants based on age, sex, smoking status and especially by NSAIDs consuming revealed that in each subgroup AA genotypes and A allele have protective effects for control subjects except in smoker subgroup. However previous studies reported, there were significant interactions between NSAIDs consuming and COX-2 variant. Some of these gene polymorphisms could modify the effect of NSAIDs in the prevention of CRC (11,24). In our study the same genotype in regular and irregular subgroups has been associated with CRC risk, therefore it could be interpret that this polymorphism did not effect on NSAIDs response. In this study may be there were some possible limitations in statistical validity of our results such as small sample size, so further association studies in a large sample size would be helped to confirm suggested correlations. Moreover other variants that were not included in this study may be involved in determining the risk of CRC and NSAIDs interaction, thus future studies are necessary.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The study was approved by Isfahan University of Medical Sciences Ethics board (approval number 392263) and written informed consent was filled and signed by all participants.

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