RESEARCH ARTICLE

Editorial Process: Submission:06/08/2022 Acceptance:10/23/2022

Role of EFNA1 SNP (rs12904) in Tumorigenesis and Metastasis of Colorectal Cancer: A Bioinformatic Analysis and HRM SNP Genotyping Verification

Elham Salem[1], Amir Keshvari[2,3], Ali Mahdavinezhad[1,4], Ali Reza Soltanian[5], Massoud Saidijam[4], Saeid Afshar[1,4]*

Abstract

Objective: Colorectal cancer is a prevalent disease with a poor prognosis and is known as a heterogeneous disease with many differences in clinical Symptoms and molecular profiles. The present study aimed to systematically evaluate the association of SNPs in miRNA binding sites of target genes that are involved in CRC angiogenesis, epithelial to mesenchymal transition, and cytoskeleton organization with tumorigenesis and metastasis of CRC. **Methods:** A case-control study was performed on 146 samples of CRC patients and 132 healthy samples. After that, the DNA of all samples was isolated by the salting-out method. Finally, the genotypes for EFNA1 SNP (rs12904) were identified by HRM (High-resolution melting analysis) method. In order to evaluate the results of genotyping, two samples from each genotype were sequenced using the sanger sequencing method. **Result:** The frequency of AA genotype and the frequency of GG for rs12904 in satge4 and other stages are different from each other (P-value <0.0001) (P-value = 0.008). Also, the frequency of AA genotype in patients with different grades is different from each other (P-value = 0.035), while the frequency of AG genotype and the frequency of GG genotype is not significantly different in patients with different grades (P-value = 0.377) (P-value = 0.284). **Conclusion:** Results of this study indicated that patients carrying the GA and GG genotypes reduced the risk of disease progression compared to the AA genotype. As a result, this polymorphism plays a key role in CRC pathogenesis and metastasis and could be used as a biomarker in molecular diagnosis and metastatic state prediction in the near future after further study of its signaling pathways and molecular mechanism.

Keywords: *EFNA1*- Colorectal neoplasms- Metastasis- Polymorphism- rs12904

Asian Pac J Cancer Prev, **23 (10)**, 3523-3531

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related death for both men and women all over the world (Siegel et al., 2018; Abdali et al., 2020). Usually, CRC is recognized by the appearance of cancerous cells in the rectum or the colon tissues. It has been mentioned that many factors such as chromosomal instability (CIN), microsatellite instability (MIN) (Lengauer et al., 1998), various mutations in proto-oncogenes(Pappou and Ahuja, 2010), tumor-suppressor genes(Armaghany et al., 2012), and also epigenetic changes in the DNA, are involved in the process of CRC tumorigenesis (Jia and Guo, 2013). Generally, it has been shown that invasion and metastasis of cancer cells could be the main cause of death in CRC and the prognosis of metastatic CRC patients was poor. (Xu et al., 2016) (Sud and Mujeeb, 2022).

The lungs and liver are the most common sites of metastasis in CRC (Hegde et al., 2008), furthermore, organs like peritoneum, brain, and bones could also be cancerous by the development and spreading of CRC cells (Vatandoust et al., 2015b). In recent decades, early diagnostic approaches and modern therapeutic options have been developed, which have led to a decrease in the CRC mortality rate. However, the majority of patients with CRC will eventually get affected by metastatic disease (Siegel et al., 2012).

In general, the metastasis process involves a complex series of events including loss of adherence to the neighboring tumor cells and obtaining migratory and invasive abilities (Yilmaz et al., 2007). In the beginning, this process requires down-regulation and up-regulation of

*[1]Department of Molecular Medicine and Genetics, Medical School, Hamadan University of Medical Sciences, Hamadan, Iran. [2]Department of Surgery, Tehran University of Medical Sciences, Tehran, Iran. [3]Colorectal Research Center, Tehran University of Medical Sciences, Tehran, Iran. [4]Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran. [5]Biostatistics and Epidemiology, School of Health, Hamadan University of Medical Sciences, Hamadan, Iran. *For Correspondence: safshar.h@gmail.com*

epithelial markers and mesenchymal markers, respectively, which is known as epithelial-to-mesenchymal transition (EMT). After that cancer cells could break away from the primary tumor and can invade by degradation of surrounding extracellular matrix proteins and basement membranes (Pantel and Brakenhoff, 2004) Followed by intravasation and survival in the blood and lymphatic vessels as well as extravasation into target organs. Eventually, invading cells can survive in a foreign microenvironment, induce angiogenesis, and develop secondary tumors (Valastyan and Weinberg, 2011). Finally, migratory cancer cells activate specific gene expression and various signaling pathways underlying sequential processes (Yilmaz et al., 2007). Accordingly, it seems that each step of this process could be a potential target for the inhibition of tumorigenesis and metastasis, so gaining attention in the field of effective anti-metastatic therapies seems to be necessary (Sartore-Bianchi et al., 2016).

In recent years, many studies have proved that microRNAs (miRNAs) play critical roles in many biological processes such as tumorigenesis, metastasis, and invasion. MiRNAs are small noncoding regulatory RNAs that are ~ 20–24 nucleotides in length (Feng et al., 2014; Li et al., 2018; Afshar et al., 2019; Mahmoudi et al., 2022). Emerging evidence reported that SNPs in the miRNA binding sites (miRSNPs) can alter the binding efficiency of miRNAs with their target mRNA. Therefore, SNPs in miRNA binding sites of protein-coding genes can lead to interruption of gene function and then cellular processes, as well as, blockage of mRNAs from repression, which result in susceptibility to various diseases like cancer (Nicoloso et al., 2010; Manikandan and Munirajan, 2014; Lee et al., 2015b). SNPs within the miRNA-binding site could be considered as potential biomarkers of tumorigenesis and clinical outcomes, as well as, diagnosis of cancer (Pelletier and Weidhaas, 2010).

Ephrin A1(EFNA1), as a member of the EFN family, is a ligand for the Eph 2 receptor (the most famous tyrosine kinase receptor)(Himanen et al., 2009). Recent studies indicated that the expression level of EFNA1 is increased in several cancers such as renal cancer, gastric cancer, and CRC(Ma et al., 2019). More ever, the EFNA1 has an essential role in tumorigenesis, angiogenesis, and invasion of cancers and can play a critical role in the prognosis of several tumors (Hao and Li, 2020).

The other aim of this study was to screen the desired genes for discovering relevance between metastasis and related biological processes including angiogenesis, EMT, and cytoskeleton organization. In the current study, we identified genes containing potential SNPs within their miRNA-binding sites which are candidates for metastasis capabilities of CRC. Eventually, rs12904, SNP in the 3'UTR of EFNA1, was selected to evaluate its role in tumorigenesis and metastasis of CRC.

Materials and Methods

Selection of Candidate Genes

Information of 19,020 approved human genes was retrieved from HUGO Gene Nomenclature Committee (HGNC) database (Yates et al., 2016), then symbol or name of each gene was searched in combination with the term "colorectal neoplasms [MeSH term]" in PubMed (https://www.ncbi.nlm.nih.gov/pubmed/). Within 19,020 HGNC genes, a total of 2,884 genes had at least two publications with this combination from 2000 to 2017.

Selection of miRNA Targets

Selected genes (2,884) in the PubMed search were evaluated for the existence of miRNA target sites in their 3′-UTR by using the TargetScan 7.1 online prediction software (http://www.targetscan.org/). As such, we have predicted which genes could be targeted by miRNA via searching for the presence of 8mer, 7mer, and 6mer sites in mRNA that matched with the seed region of each miRNA(Lewis et al., 2005).

Selection of SNPs

We screened the predicted miRNA binding sites for the presence of SNPs by a comprehensive search in the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly (https://genome.ucsc.edu/) (Kuhn et al., 2012). In this study, we considered RefSeq genes and common SNP147 as search criteria.

Gene Ontology

Finally, obtained genes were annotated by Gene Ontology biological process classifications (http://www.geneontology.org/) (Ashburner et al., 2000). The three biological processes GO terms including GO:0001525 (angiogenesis), GO:0001837(epithelial to mesenchymal transition), and GO:0007010 (cytoskeleton organization) were used to find which variants of genes involved in the CRC progression and metastasis (Figure 1).

Study population

All specimens in this study including CRC patients and healthy controls were received from patients referred to Imam Khomeini Hospital in Tehran and Shahid Beheshti Hospital in Hamadan from 2019 to 2020. This study has been approved by the Ethics Committee in Clinical Research of Hamadan University of Medical Sciences (Ethical code: IR.UMSHA.REC.1397.1029) and all patients and healthy individuals agreed to participate in this study. A total of 146 CRC patients' blood specimens (64 females and 82 males) with average age of 58.4±13.6 years and simultaneously 132 healthy individuals' blood specimens (71 females and 61 males) with a mean age of 53.7± 12. 7 years were collected (Table 1). Healthy individuals were selected considering the negative results of the pathological evaluation.

DNA extraction and Genotyping:

Blood Samples were stored at 4 ° C until DNA extraction. Genomic DNA was isolated using the salting-out method from EDTA-blood samples according to the previous study(Bartlett JMS).

To determine the genotypes for rs12904 on EFNA1 by the High-resolution melting analysis (HRM) method, we designed one set of primers using beacon designer

v7.9 (PREMIER Biosoft International, USA). The primer pairs were evaluated for specificity using NCBI Primer-BLAST(Ye et al., 2012) (Table 2). HRM analysis was accomplished on a Light cycler96 (Roche) instrument using the HOT FIREPOL Eva Green HRM Mix (Solis BioDyne, Tartu, Estonia). The protocol of thermal cycling was as follows: pre-incubation at 95°C for 720 S, 30 cycles of 95°C for 15 S, 60°C for 20 S, and 72°C for 20 S. Amplification was followed by High resolution melting analysis. In order to analyze the melting curve and detect the genotype of the samples, we assessed the results of the experiments via the Light Cycler96 precision software, version 1.1.0.1320.

In order to validate the results of HRM analysis, the genotype of some samples was evaluated with sanger sequencing. So specific primer pairs were designed for rs12904 on EFNA1 using AlleleID 6 and evaluated for specificity using NCBI Primer-BLAST. The thermal cycling protocol was as follows: initial denaturation at 95°C for 760 S followed by 32 cycles of 95°C for 45 S, 62°C for 45 S, and 72°C for 45 S, and finally 72°C for 420s. Then, PCR amplicons were subjected to Sanger sequencing(ABI 3500). Sequencing results were analyzed by CLC genomic workbench ver 11.0.1 software.

Statistical analysis

Data were analyzed using SPSS 16.0 software and only Hardy-Weinberg balance was analyzed with R software V.3.6.1. Based on the results, no deviation from the Hadry-Weinberg balance was observed in the patient group (P = 0.25), and the healthy group (P = 0.435). Comparison between two qualitative variables was performed through the Chi-square test. Adjusted OR results were performed based on the multiple-logistic regression model. P-value <0.05 was considered significant level.

Results

Achievements of bioinformatics studies

The results of the PubMed search for identifying the role of each human gene in the CRC indicated that for 19,020 HGNC genes, A total of 2,884 genes with at least two publications from 2000 to 2017 were obtained as a result of the combined search of symbol or name of each gene and "colorectal neoplasms [MeSH term]". These 2,884 selected genes were used for further evaluation by TargetScan 7.1 for the existence of miRNA binding sites in

Table 1. Characteristics and Clinical Features of the Studied Population.

Variables	Cases (n=146)		Controls (n=132)		P-value
	N	%	N	%	
Age					
	40	27.4	57	43.2	0.008
	106	72.6	75	56.8	
Sex					
Males	82	56.2	61	46.2	0.118
Females	64	43.8	71	53.8	
Addiction					
Yes	18	12.3	-	-	-
No	128	87.7	-	-	
Familial history of cancer					
Yes	65	55.5	-	-	-
No	81	44.5	-	-	
Metastasis					
Yes	29	19.9	-	-	-
No	117	80.1	-	-	
Tumor anatomical location					
Colon	67	45.9	-	-	-
Rectum	79	54.1	-	-	

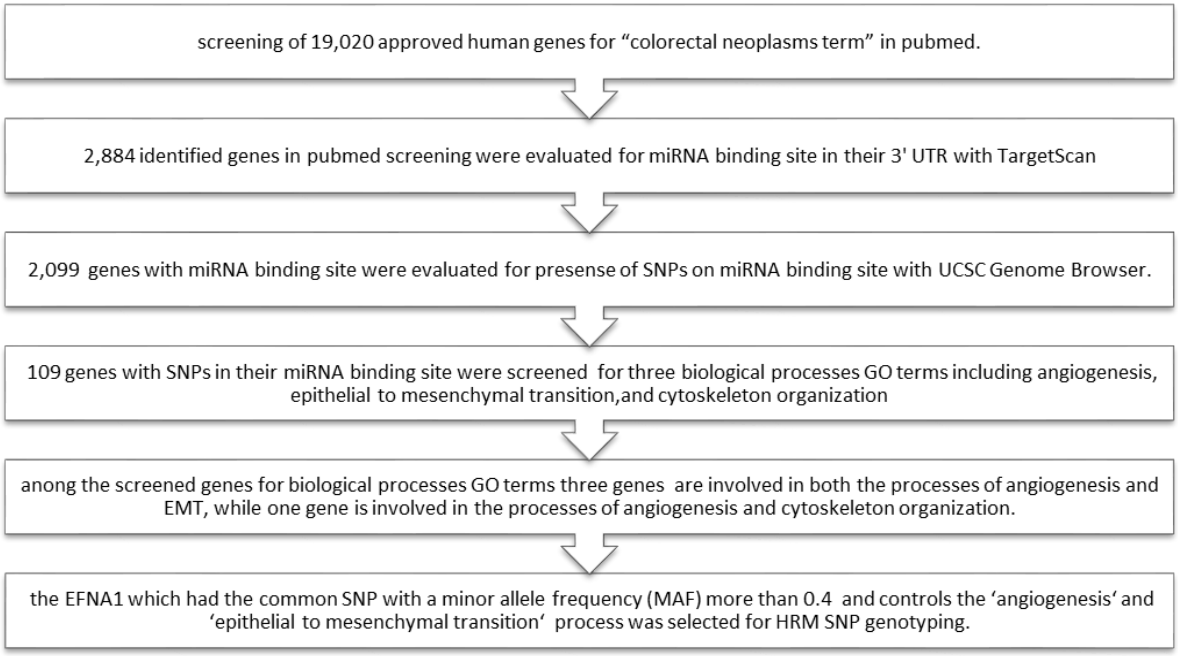

Figure 1. Schematic Diagram of Bioinformatics Analysis Workflow.

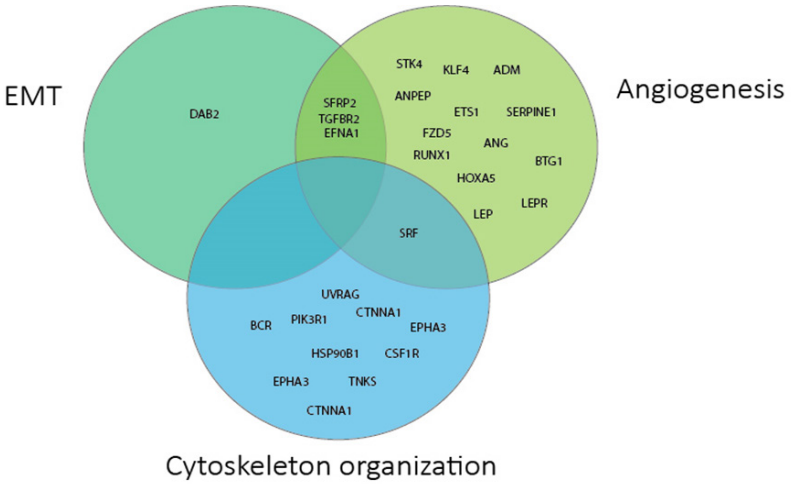

Figure 2.Venn Diagram of Identified Genes for GO Terms Angiogenesis, Cytoskeleton Organization and EMT. The three genes SFRP2, TGFBR2, and EFNA1 are involved in both the processes of angiogenesis and EMT, while the SRF gene is involved in the processes of angiogenesis and cytoskeleton organization.

Table 2. The Primers Sequence, Fragment Size, and Annealing Temperature Used for High Resolution Melting Analysis and Direct Sanger Sequencing.

Gene name	Forward primer	Reverse primer	Product size	Annealing Temperature
EFNA1(HRM)	CCACTCCCACCACAGGCATAAG	GATGGCACTGTCGGGCTGG	101	60
EFNA1(Sequence)	GTTCTACATAGCATCGGTCAC	AGTTCATCTGGGCATCCTG	499	62

the 3' UTR of their sequence. Among these genes, 2,099 genes were targeted by miRNAs and were evaluated for the presence of SNPs in their binding site for miRNAs. The results of this search have shown 121 SNPs within 109 genes. Two of 109 genes had three SNPs and 8 genes had two SNP in the binding site of miRNA and target genes. Ultimately, in order to evaluate the role of each desired gene in the progression of CRC to a metastatic state we searched 109 genes in the Gene Ontology database for three biological processes including angiogenesis, EMT, and cytoskeleton organization. Among the total 109 genes, eleven genes had the results for GO term 'cytoskeleton organization' including SRF, UVRAG, CTNNA1, PIK3R1, DES, EPHA3, CSF1R, TNKS, HSP90B1, BCR, AKAP9; seventeen of them had the results for GO term 'angiogenesis' including RUNX1, SRF, SFRP2, FZD5, TGFBR2, ANPEP, EFNA1, HOXA5, ANG, STK4, SERPINE1, KLF4, LEP, ADM, ETS1, BTG1, LEPR; four of them had the results for GO term 'epithelial to mesenchymal transition' including SFRP2, TGFBR2, EFNA1, DAB2. Tree of 109 genes including EFNA1, SFRP2, and TGFBR2 have the results for both GO term 'Angiogenesis 'and 'epithelial to mesenchymal transition' and one of them (SRF) had the results for both GO term 'Angiogenesis 'and 'cytoskeleton organization (Figure 2). EFNA1 had a common SNP with a minor allele frequency (MAF) of more than 0.4 (MAF=0.49) for the binding site of miR-200c and miR-429 in EFNA1; This common SNP was rs12904.

HRM method and Sanger sequencing for SNP detection

The rs12904 on EFNA1 was successfully genotyped via the HRM method (Figure 3) and some results were confirmed using the Sanger sequencing (Figure 4). The Sanger sequencing results in 6 DNA samples confirmed the determined genotype with HRM analysis. So, the HRM method showed enough robustness for genotype determination of rs12904 on EFNA1.

Table 3. Genotype and Allele Frequencies Distribution in CRC Patients and Healthy Samples.

	Controls No. (%)	Cases No. (%)	OR	CI 95%		Adjusted OR[a]	CI 95%[b]		Adjusted P-value
Allele									
A	96 (36%)	135 (46)	1.504	1.071	2.11	1.51	1.074	2.151	0.018
G	168 (64%)	157 (54)	Ref.			Ref.			
Genotype									
AA	15 (11.4)	35 (24)	2.59	1.254	5.338	2.69	1.285	5.631	0.009
GA	66 (50)	65 (44.5)	1.09	0.464	1.847	1.048	0.613	1.792	0.863
GG	51 (38.6)	46 31.5))	Ref.			Ref.			
Recessive Model	AA vs. GA+GG		2.463	1.261	4.808	2.618	1.335	5.128	0.005
Dominant Model	GA+AA vs. GG		1.368	0.833	2.249	0.744	0.45	1.23	0.249

[a]AR, Adjusted for Age and Sex; [b]CI, confidence interval.

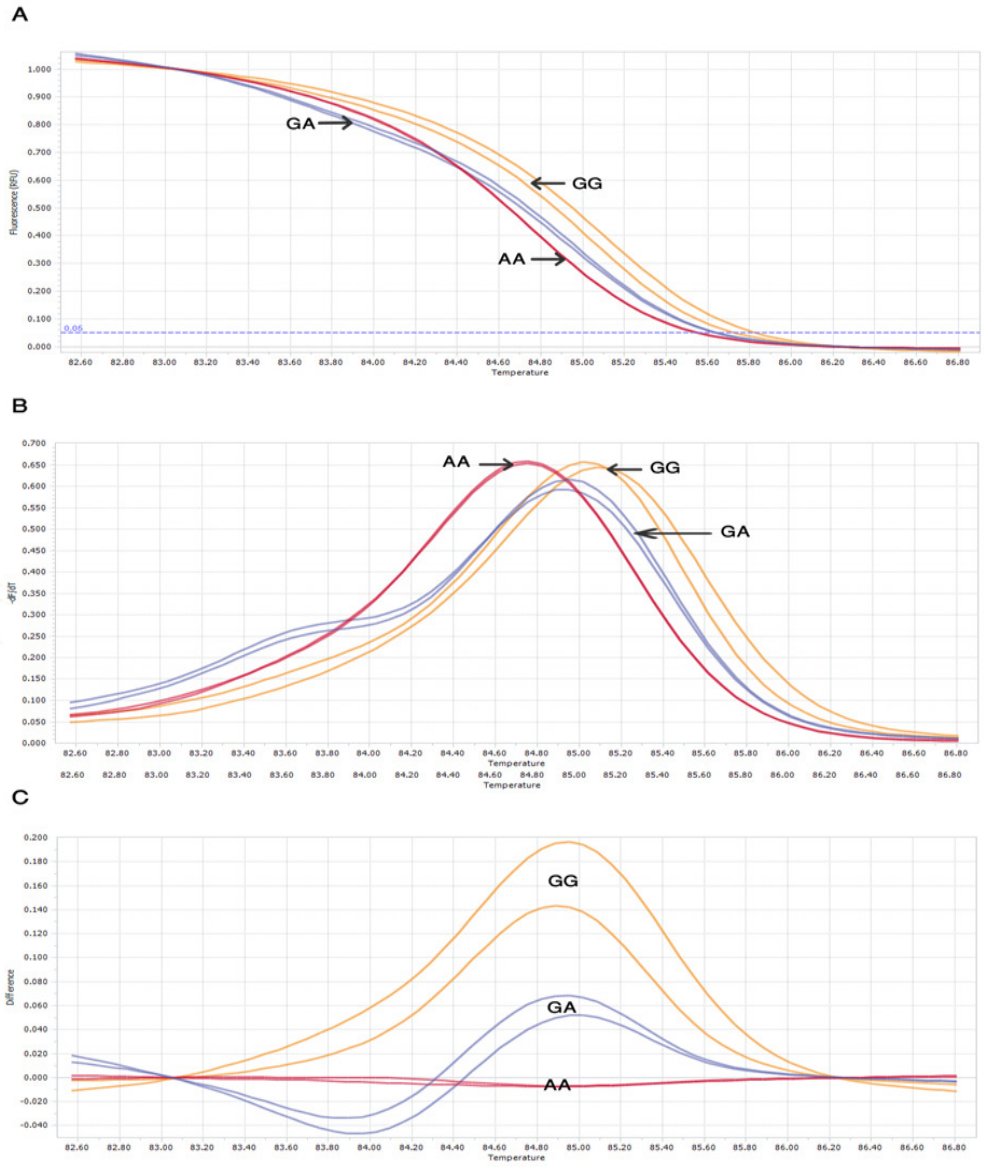

Figure 3. Genotyping the rs12904 on EFNA1 via HRM. (a) Normalized melting curve, (b) Normalized Melting Peaks, C) Difference Plot. In all plots, samples were categorized similarly to 3 genotypes including AA, GG, and AG.

A comparison of the frequency of alleles in the case and control groups indicated that allele A in the case group has a slightly higher frequency than the control group. More ever, the Odds ratio of patients carrying the A allele was 1.55 more than patients carrying the G allele (OR=1.51, 95%CI;1.07-2.15). Patients carrying the AA genotype were more susceptible to colorectal cancer compared to patients carrying the GG genotype(OR=2.69,

Table4. Genotype and Allele Frequencies Distribution in Different Dtages of CRC a

	High stages No. (%)	low stages No. (%)	OR	CI 95%		Adjusted OR[b]	CI 95% [c]		Adjusted P-value
Allele									
A	96 (53)	39 (35)	2.032	1.249	3.308	2.039	1.252	3.321	0.004
G	86 (47)	71 (65)	Ref			Ref			
Genotype									
AA	30 (33)	5 (9.1)	5.04	1.66	15.299	3.798	1.438	10.032	0.007
GA	36 (39.6)	29 (52.7)	1.04	0.488	2.227	1.155	0.549	2.432	0.704
GG	25 (27.5)	21 (38.2)	Ref.			Ref.			
Recessive Model	AA vs. GA+GG		4.918	1.777	13.61	4.987	1.797	13.836	0.002
Dominant Model	GG vs. GA+AA		0.613	0.301	1.251	0.613	0.3	1.254	0.18

[a] High stages patients (stages III and Stage IV) were compared with low stages patients (stages I and Stage II); [b]AR, Adjusted for Age and Sex; [c]CI, confidence interval.

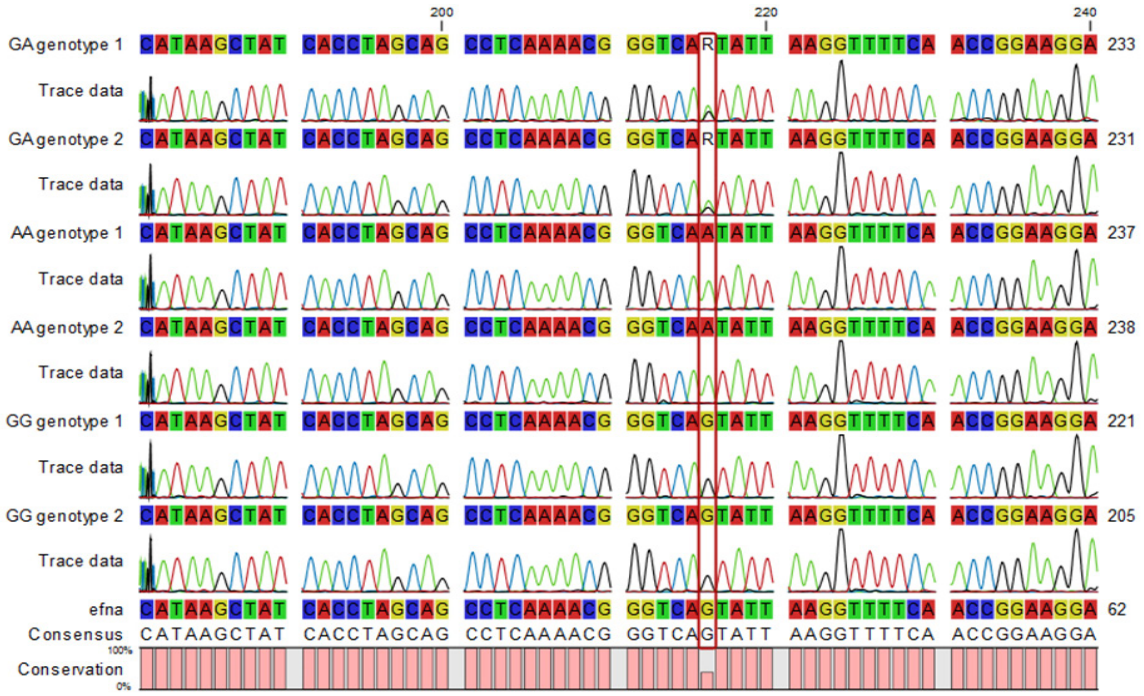

Figure 4. Sanger Sequencing Analysis for Samples. six samples which their geneotypes were identified with HRM method evaluated with sanger sequencing including 2 GA genotype , 2 AA genotype and 2 GG genotype.

Table 5. Genotype and Allele Frequencies Distribution in Different Grades of CRC a.

	High Grade No. (%)	Low Grade No. (%)	OR	CI 95%		Adjusted OR[b]	CI 95% [c]		Adjusted
Allele									
A	21 (66)	114 (44)	2.445	1.133	5.278	2.437	1.127	5.268	0.024
G	11 (34)	146 (56)	Ref			Ref			
Genotype									
AA	8 (50)	27 (20.8)	4.247	1.035	17.421	4.283	1.039	17.648	0.044
GA	5 (31.3)	60 (46.2)	1.194	0.271	5.268	1.23	0.277	5.465	0.785
GG	3 (18.8)	43 (33.1)	Ref.			Ref.			
Recessive Model	AA vs. GA+GG		3.815	1.312	11.096	3.781	1.296	11.029	0.015
Dominant Model	GG vs. GA+AA		0.467	0.126	1.726	2.203	0.592	8.202	0.239

[a]High grade patients (poor) were compared with low grades patients (well and moderately). [b]AR, Adjusted for Age and Sex; [c]CI, confidence interval.

95% CI=1.285-5.631) (Table3). More ever, There is no significant difference between the frequency of genotypes with tumor location, addiction, family inheritance, chemotherapy, and radiotherapy.

The role of rs12904 in tumor progression and grade

As seen in Table 4, AA genotype patients were at an increased chance of high-stages (satge4 and stage3) cancer (OR: 3.798, 95%CI:1.438-10.032). More ever, patients carrying the A allele had a greater risk of high-stage cancer (OR: 2.039, 95%CI: 1.252-3.321).

The risk of developing high-grade cancer in patients with the AA genotype is higher than the patients with the GG genotype (OR:4.283,95%CI:1.039-17.648). Furthermore, patients carrying the A allele had a greater chance of high-grade cancer(OR: 2.437, CI: 1.127-5.268) (Table 5).

Discussion

Cancer is a serious threat to human health. Lack of recognition of tumorigenesis, angiogenesis, and metastasis pathways, and lack of effective diagnostic and prognostic biomarkers are major obstacles in cancer management. Metastasis is the leading cause of CRC–related mortalities (Vatandoust et al., 2015a). So understanding the molecular mechanism that underlies the progression of colorectal tumors from benign to metastatic state seems to be crucial.

The origin of CRC is a combination of environmental and genetic causes. Several risk factors are dependent on lifestyle and can be diminished by performing changes in terms of physical activity and dietary; a sedentary lifestyle is thought to augment the risk of developing CRC(Odegaard et al., 2013; Van Blarigan and Meyerhardt, 2015).

In this study among all approved human genes, 109 genes with the following criteria were selected for further evaluation: All of them had (1) association with CRC (2) binding sites for miRNAs, and (3) common SNPs in the binding site of miRNA. Finally, we evaluated the role of each selected gene in three biological functions underlying metastasis including angiogenesis, epithelial to mesenchymal transition, and cytoskeleton

organization. Angiogenesis plays an essential role in several physiological and pathological processes such as metastasis (Norton and Popel, 2016). EMT is defined as a loss of polarity and cell-cell adhesion of epithelial cells and transition to mesenchymal phenotype with a high motility and invasion rate (Jolly et al., 2017). Results of Rolli et al. study indicate that the cytoskeleton organization affects the invasiveness and migration rate of tumor cells (Rolli et al., 2010).

Results of the current study indicated that EFNA1, SFRP2, and TGFBR2 had roles in both angiogenesis and EMT of CRC cells. Mao et al. in their study indicated that EFNA1 has a relationship with the angiogenesis of CRC. On the other hand dysregulation of this gene was seen in colorectal tumors compared to adjacent tissue (Mao et al., 2013). Results of two recent studies indicate that SFRP2 suppresses EMT of cervical and ovarian cancer cells through the inhibition of the Wnt/β-catenin signaling pathway (Chung et al., 2009; Duan et al., 2017). Crowley et al. in their study showed that SFRP2 induces the angiogenesis of solid tumors by activating the calcineurin/NFAT signaling pathway (Courtwright et al., 2009). Harazono et al. in their study reported that suppression of TGFBR2 through the miR-625 up-regulation leads to EMT suppression (Harazono et al., 2013). Prunier and Howe in their study showed that TGFB stimulates EMT via inducing Dab2 as an adaptor molecule (Prunier and Howe, 2005). Results of Chai et al. study indicated that SRF gene had a role in both angiogenesis and cytoskeleton organization. Results of Chai et al. study indicate that SRF (downstream mediator of VEGF) is a transcription factor which is essential for angiogenesis induced by VEGF (Chai et al., 2004).

In our study, by considering the threshold of 0.4 for MAF of the SNP, EFNA1 which regulates angiogenesis, and EMT was selected. On the other hand, rs12904 on EFNA1 has the binding site for miR-200C and miR-429. Li et al. in their survey demonstrated that rs12904 SNP of the EFNA1 gene which is located in the binding site of miR-200c is significantly related to the risk of gastric cancer. Additionally, this SNP modulated the binding of miR-200C with EFNA1 mRNA and was associated with dysregulation of EFNA1 (Li et al., 2014a). The results of Yang et al. study indicate that SNPs within the miRNA binding site of RPS6KB1 and ZNF839 mRNAs and consequently their expression levels were associated with the prognosis of CRC (Yang et al., 2017). Recent studies have drawn the attention of scientists to the relevance of EFNA1 with some cancers. Nevertheless, the association of rs12904 polymorphism with CRC is debatable. EFNA1 which is known as a member of the EFN family has a crucial role in carcinogenesis (Hao and Li, 2020). This protein binds to the membrane surface by glycosylphosphatidylinositol (GPI) and is a ligand for the EphA2 tyrosine kinase receptor(Wykosky and Debinski, 2008). As recent studies have shown that the expression of the EFNA1 gene and its receptor is increased in some cancers such as gastric, breast, bladder, and glioma. The expression level of EFNA1 was increased in colorectal tumors compared with adjacent normal tissues. Different SNPs scattered throughout the genome can be involved in the tumorigenesis of several cancers (Osian et al., 2007; Daraei et al., 2012). Rs12904 located in the 3' UTR region of the EFNA1 gene, may play an important role in miRNA binding to mRNA and the regulation of EFNA1 gene expression by miR-200c and miR-429. When the A allele replaces the G allele at position 12904, it leads to a disruption in miR-200c or miR-429 binding to mRNA and a reduction in the degradation or inhibition of the EFNA1. In 2014, Li et al. showed that EFNA1 expression was significantly modulated by rs12904. On the other hand, this SNP was associated with susceptibility to gastric cancer (Li et al., 2014b). Reports also indicate that the expression level of EFNA1 was increased in most gastric tumors. More ever, rs12904 plays a key role in the pathogenesis of gastric cancer (Lee et al., 2015a).

Due to the high prevalence of CRC in the Iranian population and the confirmation of bioinformatics data of our research on the effective role of EFNA1 in CRC angiogenesis, we decided to evaluate the association of rs12904 polymorphism on EFNA1 with CRC in case and control groups. In this study, we found that there was a significant relationship between the probability of CRC progression with the presence or absence of allele A and genotype AA in rs12904 of the EFNA1 gene. In 2012, Mao et al.illustrated that there is an association between EFNA1 rs12904 and CRC in a subgroup of the Chinese population(Mao et al., 2013).

To date, the association rs12904 of EFNA1 gene polymorphism with different stages and grades of CRC has not been investigated and the recent study is the first research about determining the association of EFNA1 gene polymorphism with metastasis of CRC.

Briefly, our data indicate a considerable association between the AA genotype of EFNA1 SNP) rs12904) and distant metastases of CRC in a group of the Iranian population. Likewise, we found a significant relationship between rs12904 and poor grade of CRC. Accordingly, EFNA1 may be considered a simple and non-invasive prognostic biomarker for CRC diagnosis.

In summary, this polymorphism plays an essential role in the pathogenesis and metastasis of CRC and could be used as a biomarker in molecular diagnosis and metastatic state prediction. These results may lead to a new perspective on the progress of targeted therapy for CRC in patients with the rs12904 in EFNA1. Nevertheless, supplementary studies investigating the relationship of EFNA1 expression level with SNP at miRNA binding site will be required.

Author Contribution Statement

MS, and SA conceived and designed the analysis. ES, AK, and SA collected the data. ES, SA, and AS contributed to analysis tools. SA, ES, AS, and AM preformed the analysis. SA, ES, MS, and AM contributed to the interpretation of the results. ES wrote the manuscript in consultation with all authors.

Acknowledgements

I would like to express my special thanks of gratitude

to Dr. Chiman Mohammadai and Mrs. Gholebagh Rahmani for contributing to data collection.

Funding Statement

The study was funded by Vice-chancellor for Research and technology, Hamadan University of Medical Sciences, Hamadan, Iran (No: 980120320).

Approval

This study was part of the MSc thesis of Elham Salem at Hamadan University of Medical Sciences.

Ethical Declaration

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors. The ethical protocol of this study was approved by the Ethics Committee of Hamadan University of Medical Sciences. (Ethical code: IR.UMSHA.REC.1397.1029) and written informed consent was obtained from all patients to participate in the study.

Data Availability

Data available on request due to privacy/ethical restrictions.

Conflict of Interest

The authors declare no conflict of interest.

References

Abdali MH, Afshar S, Pashaki AS, et al (2020). Investigating the effect of radiosensitizer for Ursolic Acid and Kamolonol Acetate on HCT-116 cell line. *Bioorg Med Chem*, **28**, 115152.

Afshar S, Sedighi Pashaki A, Najafi R, et al (2019). Cross-resistance of acquired radioresistant colorectal cancer cell line to gefitinib and regorafenib. *Iran J Med Sci*, **45**, 50-8.

Armaghany T, Wilson JD, Chu Q, et al (2012). Genetic alterations in colorectal cancer. *Gastrointestinal Cancer Res*, **5**, 19.

Ashburner M, Ball CA, Blake JA, et al (2000). Gene Ontology: tool for the unification of biology. *Nat Genet*, **25**, 25.

Bartlett JMS WAEoDfwbIBJ, Stirling D, (2003). Methods in Molecular Biology.2nd ed. Vol. 226: PCR Protocols. Totowa, NJ: Humana Press Inc.; pp 29-31.

Chai J, Jones MK, Tarnawski AS (2004). Serum response factor is a critical requirement for VEGF signaling in endothelial cells and VEGF-induced angiogenesis. *Faseb J*, **18**, 1264-6.

Chung MT, Lai HC, Sytwu HK, et al (2009). SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway. *Gynecol Oncol*, **112**, 646-53.

Courtwright A, Siamakpour-Reihani S, Arbiser JL, et al (2009). Secreted frizzle-related protein 2 stimulates angiogenesis via a calcineurin/NFAT signaling pathway. *Cancer Res*, **69**, 4621-8.

Daraei A, Salehi R, Salehi M, et al (2012). Effect of rs6983267 polymorphism in the 8q24 region and rs4444903 polymorphism in EGF gene on the risk of sporadic colorectal cancer in Iranian population. *Med Oncol*, **29**, 1044-9.

Duan H, Yan Z, Chen W, et al (2017). TET1 inhibits EMT of ovarian cancer cells through activating Wnt/beta-catenin signaling inhibitors DKK1 and SFRP2. *Gynecol Oncol*, **147**, 408-17.

Feng Y, Zhu J, Ou C, et al (2014). MicroRNA-145 inhibits tumour growth and metastasis in colorectal cancer by targeting fascin-1. *Br J Cancer*, **110**, 2300.

Hao Y, Li G (2020). Role of EFNA1 in tumorigenesis and prospects for cancer therapy. *Biomed Pharm*, **130**, 110567.

Harazono Y, Muramatsu T, Endo H, et al (2013). miR-655 Is an EMT-suppressive microRNA targeting ZEB1 and TGFBR2. *PLoS One*, **8**, e62757.

Hegde SR, Sun W, Lynch JP (2008). Systemic and targeted therapy for advanced colon cancer. *Exp Rev Gastroenterol Hepatol*, **2**, 135-49.

Himanen JP, Goldgur Y, Miao H, et al (2009). Ligand recognition by A-class Eph receptors: crystal structures of the EphA2 ligand-binding domain and the EphA2/ephrin-A1 complex. *EMBO Rep*, **10**, 722-8.

Jia Y, Guo M (2013). Epigenetic changes in colorectal cancer. *Chin J Cancer*, **32**, 21.

Jolly MK, Ware KE, Gilja S, et al (2017). EMT and MET: necessary or permissive for metastasis?. *Mol Oncol*, **11**, 755-69.

Kuhn RM, Haussler D, Kent WJ (2012). The UCSC genome browser and associated tools. *Brief Bioinform*, **14**, 144-61.

Lee J-H, Kim Y, Choi J-W, et al (2015a). Genetic variants and risk of gastric cancer: a pathway analysis of a genome-wide association study. *Springerplus*, **4**, 215.

Lee S, Choi J, Jeon H, et al (2015b). A genetic variation in microRNA target site of KRT81 gene is associated with survival in early-stage non-small-cell lung cancer. *Ann Oncol*, **26**, 1142-8.

Lengauer C, Kinzler KW, Vogelstein B (1998). Genetic instabilities in human cancers. *Nature*, **396**, 643.

Lewis BP, Burge CB, Bartel DP (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15-20.

Li J, Zou K, Yu L, et al (2018). Microrna-140 inhibits the epithelial-mesenchymal transition and metastasis in colorectal cancer. *Mol Ther Nucleic Acids*, **2018**.

Li Y, Nie Y, Cao J, et al (2014a). G-A variant in miR-200c binding site of EFNA1 alters susceptibility to gastric cancer. *Mol Carcinog*, **53**, 219-29.

Li Y, Nie Y, Cao J, et al (2014b). G-A variant in miR-200c binding site of EFNA1 alters susceptibility to gastric cancer. *Mol Carcinog*, **53**, 219-29.

Ma T-T, Wang L, Wang J-L, et al (2019). Hypoxia-Induced cleavage of soluble ephrinA1 from cancer cells is mediated by MMP-2 and associates with angiogenesis in oral squamous cell carcinoma. *OncoTargets Ther*, **12**, 8491.

Mahmoudi R, Saidijam M, Nikzad S, et al (2022). Human exposure to low dose ionizing radiation affects miR-21 and miR-625 expression levels. *Mol Biol Rep*, **49**, 1321-7.

Manikandan M, Munirajan AK (2014). Single nucleotide polymorphisms in microRNA binding sites of oncogenes: implications in cancer and pharmacogenomics. *J Integrat Biol*, **18**, 142-54.

Mao YY, Jing FY, Jin MJ, et al (2013). rs12904 polymorphism in the 3'UTR of EFNA1 is associated with colorectal cancer susceptibility in a Chinese population. *Asian Pac J Cancer Prev*, **14**, 5037-41.

Nicoloso MS, Sun H, Spizzo R, et al (2010). Single-nucleotide polymorphisms inside microRNA target sites influence tumor susceptibility. *Cancer Res*, **70**, 2789-98.

Norton K-A, Popel AS (2016). Effects of endothelial cell proliferation and migration rates in a computational model of sprouting angiogenesis. *Sci Rep*, **6**, 36992.

Odegaard AO, Koh W-P, Yuan J-M (2013). Combined lifestyle

factors and risk of incident colorectal cancer in a Chinese population. *Cancer Prev Res*, **6**, 360-7.
Osian G, Procopciuc L, Vlad L (2007). MTHFR polymorphisms as prognostic factors in sporadic colorectal cancer. *J Gastrointestin Liver Dis*, **16**, 251-6.
Pantel K, Brakenhoff RH (2004). Dissecting the metastatic cascade. *Nat Rev Cancer*, **4**, 448.
Pappou EP, Ahuja N (2010). The role of oncogenes in gastrointestinal cancer. *Gastrointestinal Cancer Res*, **2010**, S2.
Pelletier C, Weidhaas JB (2010). MicroRNA binding site polymorphisms as biomarkers of cancer risk. *Exp Rev Mol Diagn*, **10**, 817-29.
Prunier C, Howe PH (2005). Disabled-2 (Dab2) is required for transforming growth factor beta-induced epithelial to mesenchymal transition (EMT). *J Biol Chem*, **280**, 17540-8.
Rolli CG, Seufferlein T, Kemkemer R, et al (2010). Impact of tumor cell cytoskeleton organization on invasiveness and migration: a microchannel-based approach. *PLoS One*, **5**, e8726.
Sartore-Bianchi A, Loupakis F, Argilés G, et al (2016). Challenging chemoresistant metastatic colorectal cancer: therapeutic strategies from the clinic and from the laboratory. *Ann Oncol*, **27**, 1456-66.
Siegel R, DeSantis C, Virgo K, et al (2012). Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin*, **62**, 220-41.
Siegel RL, Miller KD, Jemal A (2018). Cancer statistics, 2018. *CA Cancer J Clin*, **68**, 7-30.
Sud R, Mujeeb VR (2022). Incidence of microsatellite instability, mutational burden, and actionable alterations in genes of patients with metastatic colorectal carcinoma: A Study from a Tertiary Care Hospital in India. *Asian Pac J Cancer Care*, **7**, 91-100.
Valastyan S, Weinberg RA (2011). Tumor metastasis: molecular insights and evolving paradigms. *Cell*, **147**, 275-92.
Van Blarigan EL, Meyerhardt JA (2015). Role of physical activity and diet after colorectal cancer diagnosis. *J Clin Oncol*, **33**, 1825.
Vatandoust S, Price TJ, Karapetis CS (2015a). Colorectal cancer: Metastases to a single organ. *World J Gastroenterol*, **21**, 11767-76.
Vatandoust S, Price TJ, Karapetis CS (2015b). Colorectal cancer: Metastases to a single organ. *World J Gastroenterol*, **21**, 11767.
Wykosky J, Debinski W (2008). The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting. *Mol Cancer Res*, **6**, 1795-806.
Xu P, Zhu Y, Sun B, et al (2016). Colorectal cancer characterization and therapeutic target prediction based on microRNA expression profile. *Sci Rep*, **6**, 20616.
Yang Y-P, Ting W-C, Chen L-M, et al (2017). Polymorphisms in microRNA binding sites predict colorectal cancer survival. *Int J Med Sci*, **14**, 53-7.
Yates B, Braschi B, Gray KA, et al (2016). Genenames. org: the HGNC and VGNC resources in 2017. *Nucleic Acids Res*, **2016**, gkw1033.
Ye J, Coulouris G, Zaretskaya I, et al (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, **13**, 134.
Yilmaz M, Christofori G, Lehembre F (2007). Distinct mechanisms of tumor invasion and metastasis. *Trends Mol Med*, **13**, 535-41.