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The Significant of miR-196a2 C>T Single Nucleotide Polymorphism and Serum Levels of Interleukin-1β (IL-1β) and Intrleukin-6 (IL-6) in Colorectal Cancer

Noor Abdal-zahra[1], Subhi Jawad Hamza[2], Haidar Ahmad Shamran[3], Qasim S. Al-Mayah[3]
[1]*Usul Aldeen University College/Iraq*
[2]*College of Biotechnology/ Al-Nahrain University/Iraq*
[3]*Medical Research Unit/ College of Medicine/ Al-Nahrain University/Iraq*

Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality. Beside the environmental factors, individual's genetic configuration and inflammatory events could have a crucial role in the incidence of this malignancy.

This study aimed to evaluate the role of miR-196a2 C>T polymorphism and serum levels of IL-1β and IL-6 as risk factors for CRC.

This case-control study included 55 patients with confirmed CRC and age- and sex-matched 30 apparently healthy individuals as controls. Blood samples were collected from each subject, and DNA was extracted from leukocyte. The gene corresponding miR-196a2 C>T polymorphism was amplified with specific primers, and genotyping was achieved by direct sequencing. Sera were used to measure IL-1β and IL-6 levels via enzyme-linked immunosorbent assay (ELISA).

The results show that Allele T of miR-196a2 C>T polymorphism was significantly more frequent among CRC patients than controls adjusted for demographic factors and comorbidities (OR=2.447, 95%CI=1.142-5.241, P=0.021). Furthermore, in the dominant model, TT+TC combination was more frequent in CRC patients than control with a significant difference (OR=2.603, 95%CI= 1.013-6.685). Serum levels of IL-1β and IL-1 were significantly higher in patients than controls (P<0.05).

These data suggest the role of miR-196a2 C>T as a predisposing genetic factor for CRC. Proinflammatory cytokines of high levels, particularly, IL-1β and IL-6, may increase the individual's susceptibility for CRC.

Keywords: colorectal cancer. microRNA, miR-196a2 C>T, interleukin-1β, interlukin-6

INTRODUCTION

Colorectal cancer (CRC) is the fourth common cancer in women and men worldwide, with an estimated over 1 million incidence from new cases per year. The mortality-to-incidence rate ratio of CRC is approximately 0.51, which accounts for cancer-related health burdens a significant amount [1]. The incidence and mortality of CRC could be minimized if individuals at the disease risk are detected and treated early. Risk factors identification associated with CRC represents a promising strategy for facilitating the early detection at-risk individuals [2].

Environmental factors number that influence the susceptibility to CRC have been identified. These include smoking of tobacco, red meat consumption, alcohol use and fibrous food low intake [3]. Since, these factors of risk in nature are modifiable, their effects on risk are CRC are likely complex. Exposure to these factors of risk alone is inadequate to result in carcinogenesis of colorectal.

The implication of these risk factors of environmental in improving cancer risk prediction and medical screening is likely limited.

Discovery of microRNAs (miRNAs) has revolutionized the understanding of gene regulation mechanisms [4]. MiRNAs are short, single-stranded, non-coding fragments that regulate gene expression via base pairing with either mRNAs at 3'-untanslated region (3'-UTR) or an open reading frame (ORF). This base-pairing results in translational suppression or cleavage and degradation of mRNA, respectively [5]. Single nucleotide polymorphisms (SNPs) in miRNAs genes may change the expression and/or maturation of the corresponding miRNA [6]. Accordingly, the expression of many gene could be altered which influences a large number of vital body function such as immune response [7]. The miR-196a2 gene is located on chromosome 12q13.13 and are involved in the regulation of many mRNAs. The SNP rs1164913 in this gene was found to be associated with several malignancies such as lung cancer and hepatocellular carcinoma [8].

These evidence has demonstrated that patients infected in chronic inflammation in bowels have an CRC risk. Various mediators and inflammatory cells produced during chronic inflammation are orchestrated through different pathways of molecular signaling and lead to the a microenvironment formation in tumorigenesis favor [9]. Pro-inflammatory cytokines, particularly, IL-1β and IL-8 are known to induce inflammatory response when their levels are high enough, and thus may associated with CRC [10-11].

The current study aimed to evaluated the role of miR-196a2 and serum levels of IL-1β and IL-6 with the susceptibility to CRC among Iraqi patients.

SUBJECTS AND METHODS

The Study Population

Patients attending hospitals which undergo CRC from July 2016 to November 2016 were eligible for this study. Three hospitals in Iraq were included: Baghdad Medical City, AL- Amal Hospital and AL-Amamain AL-Kadhamain Medical City. Ethical clearance to conduct the research was obtained from these hospitals. Patient's selection were accomplished with the surgeon's assistance.

Fifty-five patients included to be investigated in this study. All had colorectal cancer of stages and different grade (31

men and 24 women) with a mean age of 53.8 years (range between 16 -75). Data were collected by direct interview with the patients, seeking his\her hospital record and previous medical reports.

Patients claims were followed as an information alternative source when this previous medical reports were not available.

Thirty age- and sex-matched apparently healthy subjects were randomly selected to represent the controls. Individuals who diagnosed with cancer were excluded from this group. Informed consent forms from patients as well as controls were taken which include age, sex, smoking, diabetes mellitus (DM), body mass index (BMI), residence, and first or second relative family history of CRC.

Samples

Five ml of venous blood taken from each participant; 2 ml of which is kept in EDTA tube (used in study of genetic and kept at -20° C) and the reminder 3 ml in a plain tube which was subjected to centrifugation where the serum was obtained and preserved at -20°C until used.

DNA Isolation and Genotyping

DNA was isolated from blood samples using ready kit (gSYNC^{TM} DNA Mini Kit Whole Blood Protocol/ Geneaid/ Taiwan) according to the manufacturer's instructions. The primers used for amplification of miRNA containing 196a2 C> T SNP were forward primer 5'-CCC CTT CCC TTC TCC TCC AGA TA-3', and reverse primer 5'-CGA AAA CCG ACT GAT GTA ACT CCG-3' [12]. The reaction tube contains, 0.4 µmol-l reverse primer, 0.4 µmol-l forward primer, DNA template (2 ng), Taq DNA polymerase (0.05 µl), 4 mmol-l MgCl2, dNTPs (dATP, dCTP, dGTP and dTTP, 0.4 mmol each. A final volume of 50 µl that obtained by adding ddH2O. The conditions of PCR is follows: after denaturation for 5 min at 94°C, 35 cycles for 30 sec for 94°C, 72°C for 1 min and 58°C for 30 sec. The extension step of final for 7 min at 72°C. Products PCR were see gel electrophoresis, stained with bromide of ethidium for 20 min and examined using U. V. trans illuminator with camera.

The products of amplified determined by comparison with a commercial 1000 bp ladder. After the targeted regions amplification, 35µl of product PCR along with primers, were sent abroad to Macrogen Company/ Korea for direct sequencing.

Alignment conducted using alignment tool, BLASTn for nucleotide sequence. This available online at the National Center Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov). Chromas pro software used to analyze the sequences.

Quantitative determination of serum concentration of IL-6 and IL-1β

Sandwich ELISA technique (MyBiosource, China) was used for serum level estimation of IL-6 and IL-1β for fifty-five colorectal cancer patient samples and thirty controls following manufacturer's instructions.

Analysis of statistical

The Statistical Package for the Social Sciences (SPSS, version 14) was used to analysis of statistical. Risk association between the RCR susceptibility and genotype was estimated by the adjusted odds ratio (OR) calculation and using logistic regression to 95% confidence intervals (CI). For this analysis, subjects which homogenous for the wild type allele were considered a reference, and polymorphisms as dependent variables, age, sex, family history, residence, BMI. And smoking as co-variables in the model.

Chi- square used for testing the deviation from H-W equilibrium, and for testing different alleles of distribution between patients and control. Data for serum levels of cytokines and were analyzed with independent t-test was used to compare means. A P-value of 0.05 was considered significant.

RESULTS

Demographic and Comorbidities Data of the Study Population

Table 1 shows the demographic and comorbidities for CRC patients and controls. Two factors appeared to have a significant association with CRC. These were DM and family history. More than a half patients were diabetic compared to only 3.33% of controls (P=0.001). Likewise, 38.18% of patients had a family history of CRC compared to 6.67% of controls who had such a history (P=0.006).

Table (1): Demographic and comorbidities data for patients of colorectal cancer and control.

Factor	CRC Patients(55)	Controls (30)	*P*-value	OR(95%CI)
Age, years	53.8±8.93	43.41±9.1	0.074	……..
Diabetes				
No	25(45.45%)	29(66.67%)	0.001	1.0
Yes	30(54.55%)	1(3.33%)		34.8(4.42-273.84)
Dwelling				
Rural	15(27.27%)	3(10%)	0.074	1.0
Urban	40(72.73%)	27(90%)		3.37(0.891-12.79)
Smoking				
Never	28(50.91%)	12(40%)	0.337	1.0
Ex\current	27(49.09%)	18(60%)		1.55(0.631-3.83)
Family history				
No	34(61.82%)	28(93.33%)	0.006	1.0
Yes	21(38.18%)	2(6.67%)		8.647(1.86-40.1)
Sex				
Male	31(56.36%)	18(60%)	0.746	1.0
Female	24(43.64%)	12(40%)		1.16(0.47-2.87)

Gel electrophoresis of PCR products

DNA was extracted from 55 blood samples obtained from CRC patients as well as 30 blood samples from healthy control. Using specific primers, the amplification of miRNA gene corresponding to miR-196a2 C>T was carried out. Gel electrophoresis of PCR products are shown in figures (1)

Genotyping

Sequencing of miR-196a2 C>T revealed three genotypes in both CRC patients and controls which are CC, CT and TT(Figure 2). The distribution of the three genotypes was in accordance with HWE.

Table 2 shows the frequency of these genotypes in patients and controls. According to the statistical analysis, there is no significant differences between CRC patients and controls regarding the frequency of miR-196a2 C>T genotypes. Stratifying the genotypes according to inheritable models revealed higher frequency of TT+TC among CRC patients than controls (52.73% vs. 30%) with a significant difference (OR=2.603, 95%CI=1.013-6.685, P=0.047). Likewise, allele T was more frequent among CRC patients than controls (35.45% vs. 18.33%) with a significant difference (OR=2.447, 95%CI=1.142-5.241, P=0.021).

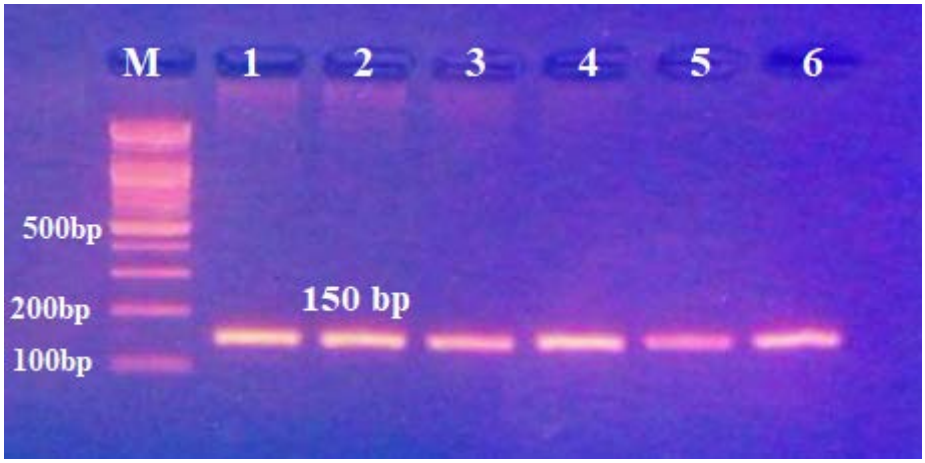

Figure (1): Gel electrophoresis for miR-196a2 C>T PCR products visualized under UV light. M: 1000 bp marker; lanes 1-6: from blood of CRC patients, lane 9: from control blood. The product size is 150 bp.

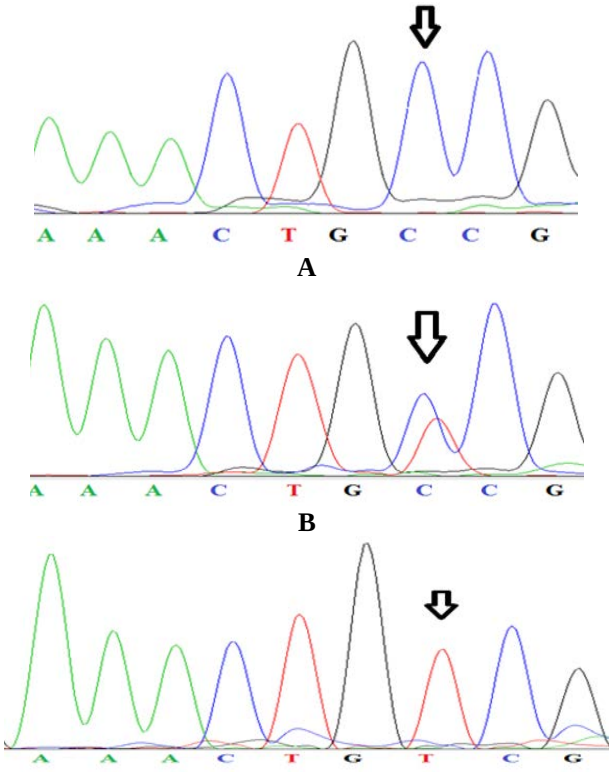

Figure (2): DNA sequencing miR-*196a2 C >T* SNP (rs11614913). A: wild type homozygous genotype CC, B: heterozygous genotype CT, C: homozygous mutant genotype TT.

Table 2: Frequencies of different genotypes and alleles for miR-*196a2* C>T in colorectal cancer patients and controls.

miR-196a2 rs11614913	**Cases N=55**	**Control N=30**	***P*-value**	**OR(95%CI)**
Genotypes				
CC	26(47.27%)	21(70%)	0.122	1.0
CT	19(34.54%)	7(23.33%)	0.139	2.192(0.775-6.203)
TT	10(18.18%)	2(6.67%)	0.092	4.038(0.796-20.477)
HWE *P*	0.69	0.227		
Dominant Model				
CC	26(47.27%)	21(70%)	0.047	1.0
TC+TT	29(52.73%)	9(30%)		2.603(1.013-6.685)
Recessive Model				
TT	10(18.18%)	2(6.67%)	0.162	1.0
CC+CT	45(81.82%)	28(93.33%)		3.111(0.635-15.253)
Alleles				
C	71(64.55%)	49(81.67%)	0.021	1.0
T	39 (35.45%)	11(18.33%)		2.447(1.142-5.241)

Serum levels of IL-1β and IL-6

Serum levels of both IL-1β and IL-6 were significantly higher in CRC patients (9.07±1.94 pg/mL and 12.028±3.12 pg/mL and respectively) than control group (5.3±1.3 pg/mL and 9.18±2.2 pg/ml respectively).

DISCUSSION

In the current study, T allele was found to be significantly increased the CRC risk among Iraqi patients with CRC (OR=2.447, 95%CI=1.142-5.24, P= 0.021). This implies that T allele carriers are at about 2.5-fold increased risk of CRC compared with C allele carriers. Such result is comparable to that of Kupcinskas *et al*. who found that the polymorphism of miR-196a-2C>T was significantly associated with CRC risk [13]. Similarly, Li *et al*. Indicated that miR-196a-2 C>T increased the susceptibility to CRC. However, in the subgroup analysis by ethnicity, they observed that this polymorphism to C allele is associated with a high risk of colorectal cancer in Asian populations [14]. Moreover, no association was indicated in Caucasian populations. Chen *et al*. did not find any association between this polymorphism and CRC among Chinese population [15]. In a recent meta-analysis including 84 articles with 38802 cases and 41541 controls, Liu *et al.* found that miR-196a-2 C>T conferred a decrease in susceptibility to lung cancer and hepatocellular carcinoma, but not for breast cancer or CRC [7]. This inconsistency in the results was frequently encountered, which potentially arises from different allele frequencies among ethnic groups, different cancer types, sample size, and sample collection and preservation [16-18].

The mechanisms beyond this effect is not fully understood; however, evidence showed that the main target of miR-196a-2 is HOX gene family [17]. HOX gene expression of studies in various cell lines of colon cancer HOX genes upregulation [19]. According to Liao *et al*. HOXB7 overexpression contributes to proliferation and tumorigenesis in cells of colorectal both in vitro and in vivo. That due to its role in regulation of cell cycle, via cyclin D1 upregulation and p21Kip1 downregulation through an activated phosphatidylinositol-3-kinase (PI3K)–protein kinase B (Akt) signaling pathway and mitogen-activated pathway kinase (MAPK) [20]. Supporting these evidences, miRNA-196a was found to control melanoma-associated genes by regulating HOXC8 expression [21].

Chronic inflammation is responsible for approximately 15% of all epithelial tumors, and the correlation between prolonged inflammation and CRC was documented in patients with inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease [22-26]. Both of IL-1β and IL-6 are powerful pro-inflammatory cytokines, and were found to be significantly elevated in CRC in the current study.

Generally, IL-1 is secreted by various type of cells, particularly from myeloid cells, upon inflammatory or stress conditions. It was shown that IL-1β level is increased in most cases of cancer, especially colon cancer [14]. Indeed, the secreted IL-1β from the local epithelial cells induces the secretion of growth factors that promote cell proliferation and cancer development. Moreover, when sufficient amount of IL-1β are secreted, they induce myeloid derived suppressor cells (MDSC)-mediated suppression that either inhibits anti-tumor immunity or obscure it. In either cases, there will be a progression in tumor growth [10]. In supporting this concept, Gunter *et al.* found that a polymorphism in IL-1β gene which accompanied with increased serum levels of IL-1β has been associated with high risk of CRC [27].

IL-6 is a cytokine of proinflammatory secreted by adipose tissue and immune cells [11]. It is considered as an important factor of tumor promoting in various types of human cancer including melanoma, glioma, lymphoma, as well as pancreatic, breast, ovarian, renal, prostate, and of course colorectal cancer. Various studies found an increased IL-6 expression in patients with colorectal cancer, where levels of IL-6 are elevated in the patients serum and in tumor tissue itself [28]. According to Knüpfer and Presis, expression of IL-6 could be associated with tumor stage, metastasis, size, and patient's survival with colorectal cancer [29]. Willet *et al.* demonstrated that high

levels of this cytokine is associated with poor prognosis in patients of colorectal cancer receiving chemotherapy of bevacizumab [30]. Due to the IL-6 expression correlation with colorectal cancer prognosis and the increased IL-6 expression in patients with IBD, IL-6 is thought to act as a link between tumor development and chronic inflammation.

Collectively, these data indicate the role of miR-196a-2 C>T polymorphism and a genetic predisposing factor for CRC. High IL-1β and IL-6 levels could also involve in the susceptibility to the cancer through a creation of prolonged inflammatory environment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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