Effects of VEGF Gene Polymorphisms on BEV Responsiveness in a Sample of Iraqi Colorectal Cancers Using HRM - PCR

Alaa Tariq[1], Wiaam Ahmed Al-Amili[1], Ahmed Zuhair Abdulhameed Alsammarraie[2], Waleed Jassim Mohammed Alserartee[3], Sarah Mohssen Mohammed[4]

[1]Institute of Genetic Engineering and Biotechnology for Post Graduate Studies, University of Baghdad, Iraq, [2]Medical oncology specialist (MBChB FICMS), Oncology Teaching Hospital Baghdad, Iraq, [3]Clinical Oncologist, Alamal National Hospital, Medical City Complex, Iraq, [4]Laboratories Department, Medical Technology B.ScPG Dip, Ministry of health, Iraq

Abstract

Background: Vascular endothelial growth factor (VEGF) have a vital role in the molecular genetic events of angiogenesis and vasculogenesis, so it is involved in the development of cancer. Single nucleotide polymorphisms (SNP) in VEGF gene has been announced as a risk factor in colorectal cancer. Bevacizumab (BEV) is an angiogenesis inhibitor that curb the binding of VEGF to its receptors obstructing the angiogenesis process. **Objective:** The ideal goal of ongoing study lies in revealing the effect of rs699947 (-2578 C/A (and rs833061 (-460C/T(polymorphisms in the promoter of VEGF gene on the development of colorectal cancer and on the BEV responsiveness in a sample of Iraqi patients using High Resolution Melting Analysis (HRM) analysis. **Methodology:** Venous blood samples were collected from 25 colorectal cancer patients with response to BEV treatment and 25 with BEV resistant and 25 apparently healthy individuals as control group who matched with patients in age and gender. **Results:** AA and CA polymorphisms A allele of rs699947 (-2578 C/A) and TT and CT polymorphisms and T allele of rs833061 (-460C/T(were represent a risk factor on the occurrence of the colorectal cancer. It has been found that CC and CA polymorphism of the VEGF - 2578 C/A and CT genotype of the VEGF -460 C/T polymorphism might be a predictive factors of responsiveness to BEV chemotherapy in CRC patients. **Conclusion:** These outcomes confirm the essential role that VEGF polymorphisms play in the occurrence of CRC and the correlation between SNPs in VEGF promoter region and the BEV responsiveness. With this, further research and investigation of VEGF polymorphisms could allow for its use in identifying risk factors for the development of CRC and increasing its predictive value for anti-VEGF cancer therapies.

Key word: *VEGF Gene, Polymorphisms, BEV Therapeutic, Colorectal Cancers, HRM – PCR.*

Introduction

Angiogenesis, the growth of new blood vessels from preexisting vessels, is an important process in physiological conditions, such as wound healing and tissue regeneration.[1] or pathological conditions, such as heart disease and tumor formation. [2,3]. Vascular tumor generation (angiogenesis) is an essential process play an important role in the occurrence of tumor and metastases. It is controlled by the complex and coordinated procedures of pro-angiogenic factors and their receptors that become upregulated during tumor formation[4]. Cancer cells are characterized by their ability to secrete many defective growth factors that contribute to stimulating the formation of neoplastic vessels. Among them, the VEGF represents one of the major powerful endothelial cell generators, and one of the most important triggers for vascular formation in CRC[5, 6]. Vascular endothelial growth factor (VEGF) is a signaling protein that contributes to the growth of new blood vessels. VEGF plays an important role in the process of blood flow to cells and tissues when there is hypoxemia caused by poor blood circulation. Thus, VEGF have an intrinsic role in both vasculogenesis and the formation of tumor blood vessels[7]. On the other hand, inhibiting the process of formation of neoplastic hemangiomas contributes to reducing tumor formation.

One of the angiogenesis inhibitors is Bevacizumab (BEV), which represents a humanized antibody that is used to target the VEGF gene[4]. For this treatment is used with many types of cancer, including colorectal cancer[8]. Many single-nucleotide polymorphisms (SNPs) were demonstrated to regulate VEGF expression. Of that, rs833061 and rs699947 are two substantial SNPs located in the VEGF promoter gene, which might affect on promoter activity.[9,10]

Methods

This study was carried out on a total of 75 subjects included 50 Iraqi patients with colorectal cancer who attended Alamal National Hospital and Oncology Teaching Hospital Baghdad during the period extended from the first of November /2019 to the end of January/2020, with age ranged from 25-86, and 25 apparently healthy voluntaries matched the patients group with age and gender.

Samples collection

Amount of three ml of venous blood was withdrawn from each subject under aseptic conditions after informing them about the aim of the research and filling in the questionnaire forms by them. Two ml of blood was placed in EDTA tube (1.5 mg/ml) and kept at -20 Cº to be used in molecular study.

Genomic DNA was automatedly extracted from the whole blood samples of all subjects by using Blood DNA Extraction Kit 200 (MagPurix/Taiwan). The MagPurix technology is a state of the art platform that uses magnetic beads to extract nucleic acids from samples. The platform commits a truly walk-away automation in nucleic acid purification from samples to results. The purification process contains steps of lysis, binding, washing and elution. After genomic DNA was extracted, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

The qRT-PCR-HRM assay with specific primers (Alpha DNA / Montreal , Canada) designed with the Prime 3 software as shown in Table 1, was used to identify SNPs rs833061 (- 460 C / T) and rs699947 (- 2578 C / A) in the promoter region of the VEGF gene to investigate effects of VEGF polymorphism on CRC occurrence and the clinical response to BEV of CRC patients, using a specific primers and positive and negative controls that ensures a high degree of specificity. The program of HRM assay shown in table 2.

Table 1 : Sequence of primers that are used within this study

SNP	Primers	Primer sequences 5' - 3'	Product Size
rs699947	F	5' TTCCCATTCTCAGTCCAT 3'	88bp
	R	5' CAGTCAGTCTGATTATCCA 3'	
rs833061	F	5` TCTGTGTGGGTGAGTGAG 3`	85bp
	R	5' TATTGGAATCCTGGAGTGA 3'	

Table 2 : HRM program

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denature	95 °C	30 seconds	35
Anneal	56 °C	60 seconds	
Extension	72	1 minute	
Melting curve Analysis	72-95 °C	0.3°C/S	1

Result and Discussion

In order to estimate the effects of SNPs of VEGFA gene on response to BEV therapeutic in Iraqi colorectal cancer, results of rs699947 and rs833061 SNPs were compared. The findings of this study presented that AA and CA genotypes, and A allele of VEGF rs699947 were significantly increased the risk of colorectal cancer in patients group rather than control group

(O.R. = 1.766 ; p = 0.0001). While, CC polymorphism and C allele was elevated in control group significantly (P≤0.01) when compared with patients group, considering a protective factor for them (table 3). This result is consistent with what has been reached by Jannuzzi *et al.* (2015) ,who found that −2578A>C was altogether connected with CRC chance.[11]

When comparing the group of patients who are response to treatment of BEV with those who were resist to this treatment , it has been found that CC and CA polymorphism of the VEGF - 2578 C/A was highly significant associated with BEV responsiveness (O.R =1.307; p= 0.0006) and C allele frequency (0.52) was significantly higher in response group , pointing to the BEV response marker in CRC patients was CC and CA genotype and C allele (table 4). This is completely consistent with the research findings of Wang *et al*. (2015), as he showed that CC genotype has a close relationship of the therapeutic response to BEV treatment. [12]

An opposite results was obtained in rs833061 SNP analysis , that TT and CT genotypes increase in patients group than in control group(O.R. = 1.397 ; P=0.0001) and T allele was significantly higher in patients group, indicating that TT and CT genotypes and T allele were a risk marker for the occurrence of CRC , while , CC genotype and C allele was significant higher in control group to be a protective factor for them (O.R.=1.754 ; P = 0.0001) as illustrated in table 5. Whereas, Jannuzzi *et al.* (2015) pointed that the distribution of -460 C> T between patients and controls did not differ significantly. [11]

In other hand , CT genotype of the VEGF -460 C/T polymorphism might be predictive factors of responsiveness to BEV chemotherapy in CRC patients (1.703 , p= 0.0001) as shown in table 6 . In addition , C allele was significantly (P= 0.015) associated with BEV responsiveness. This is in line with the Wang *et al.* (2015) conclusion in identifying CT genotype as a therapeutic response factor to the BEV treatment. [12]

Blood vessels are important for tumores to get its nutrition and oxygen to be developed and grow.A major mediator of angiogenesis in cancer is VEGF, it has the powerful action to boost the angiogenesis. [13] BEV targeted VEGF, enervate or blocks the interaction between VEGF and its receptor, In doing so, it would be discourage endothelial cell proliferation, ending in knock down the angiogenesis in tumorigenesis . Finally, suppression of angiogenesis could prohibit the growth of

tumors and enhance the therapeutic efficiency. [14]

Since CRC is one of the most malignant cancers in the large intestine, it has the highest incidence and mortality. [15]

The therapeutic efficacy of the response to BEV therapy is divers in patients who receiving the same BEV therapy. This event may be determined by the individual genetic variation. According to reports published , single nucleotide polymorphisms in VEGFA gene plays a crucial role in the development of tumors and cancer , of them CRC. The therapeutic competence of BEV may influenced by the SNPs in VEGF gene. [16]

The most two vastly investigated SNPs in the promoter region of VEGFA gene are rs833061 (−460C>T) and rs699947 (−2578C>A). Examinations specify that these SNPs could influence the promoter activity of the VEGFA gene , and afterward change the VEGF expression.

Conclusion

As a goal of BEV treatment, abnormal VAGFA accumulate may lead to angiogenesis, leading to therapeutic disappointments. Although VEGF has been targeted by BEV, the polymorphisms at the promoter region of VAGFA gene may cause accumulation of its product and lead to angiogenesis and subsequently may fundamentally impact the helpful adequacy of BEV chemotherapy. [17,18]

Conflict of Interest: None

Funding: Self

Ethical Clearance: Not required

Table 3 : Distribution of genotypes and allele frequency of SNP rs699947 in difference groups

Group	Genotypes			O.R.	P-value
	CC	**CA**	**AA**		
Patients (No = 50)	12 (24.00%)	6 (12.00%)	32 (64.00%)	1.766	0.0001 **
Control (No = 25)	17 (68.00%)	2 (8.00%)	6 (24.00%)	1.713	0.0001 **
P-value	0.0001 **	0.348 NS	0.0001 **	---	---

Allele frequency		
Group	C	A
Patients	0.30	0.70
Control	0.72	0.28

** (P≤0.01)-HS.

Table 4 : Relationship between rs699947 SNP genotypes and allele frequency with BEV responsiveness.

Group	Genotypes			O.R.	P-value
	CC	CA	AA		
Resistant patients (No = 25)	1 (4.00%)	2 (8.00%)	22 (88.00%)	1.893	0.0001 **
Response patients (No = 25)	11 (44.00%)	4 (16.00%)	10 (40.00%)	1.307	0.0006 **
P-value	0.0001 **	0.0473 *	0.0001 **	---	---

Allele frequency		
Groups	C	A
Resistant patients	0.08	0.92
Response patients	0.52	0.48

* (P≤0.05)-S., ** (P≤0.01)-HS.

Table 5 : Distribution of Genotype and allele frequency of SNP rs833061 in difference groups

Group	Genotypes			O.R.	P-value
	CC	CT	TT		
Patients (No = 50)	24 (48.00%)	24 (48.00%)	2 (4.00%)	1.397	0.0001**
Control (No = 25)	16 (64.00%)	9 (36.00%)	0 (0.00%)	1.754	0.0001 **
P-value	0.0218 *	0.0457 *	0.308 NS	---	---

Allele frequency		
Group	C	T
Patients	0.72	0.28
Control	0.82	0.18

* (P≤0.05)-S. , ** (P≤0.01)-HS.

Table 6: Relationship between rs833061 SNP genotypes and allele frequency with BEV responsiveness.

Group	Genotypes			O.R.	P-value
	CC	CT	TT		
Resistant patients (No = 25)	12 (48.00%)	11 (44.00%)	2 (8.00%)	1.658	0.0001 **
Response patients (No = 25)	12 (48.00%)	13 (52.00%)	0 (0.00%)	1.703	0.0001 **
P-value	NS	0.0225 *	0.0489 *		
Allele frequency					
Genotype	C		T		
Resistant patients	0.70		0.30		
Response patients	0.74		0.26		
*** (P≤0.015)-S., ** (P≤0.01)-HS.**					

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