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Association between *EGF* and *VEGF* functional polymorphisms and sporadic colorectal cancer in the Malaysian population

T.P. Lau[1], A.C. Roslani[2], L.H. Lian[3], P.C. Lee[4], I. Hilmi[5], K.L. Goh[5] and K.H. Chua[1]

[1]Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
[2]Division of General Surgery, Department of Surgery, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
[3]Casa Vista, Jalan Penaga, Kuala Lumpur, Malaysia
[4]School of Science and Technology, Universiti Sabah Malaysia, Kota Kinabalu, Sabah, Malaysia
[5]Division of Gastroenterology and Hepatology, Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Corresponding author: K.H. Chua
E-mail: khchua@um.edu.my

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ABSTRACT. Growth factors are polypeptides that are critical for the initiation, progression, and metastasis of cancer. Most tumor cells are capable of synthesizing particular growth factors leading to constitutive pathway activation in these cells through autocrine signaling. Epidermal growth factor (EGF) is a potent mitogenic peptide that exerts direct effects on the proliferation and differentiation of tumor cells in carcinogenesis. By contrast, vascular endothelial growth factor (VEGF) is vital for the invasion and metastasis of neoplasms through the formation of new blood vessels from mature endothelial cells. In this study, we investigated the association between functional polymorphisms of both

the *EGF* and *VEGF* genes and colorectal cancer (CRC) susceptibility. A total of 130 CRC patients and 212 healthy controls were recruited for this case-control study. Genotyping of genetic variants was conducted via real-time polymerase chain reaction (PCR) amplification with allele-specific TaqMan probes. None of the genotypes of the *EGF* +61 A>G and *VEGF* +936 C>T variants was significantly associated with CRC susceptibility among the Malaysian subjects evaluated ($P > 0.05$). The observed frequency distributions of the *EGF* +61 A>G polymorphism genotypes showed ethnic heterogeneity, which was not the case for the *VEGF* +936 C>T genotypes. In conclusion, no positive correlation between these functional polymorphisms and CRC risk was found in this Malaysian population. Studies of the *EGF* and *VEGF* genes and CRC susceptibility are scarce, and the results reported thus far differ from one population to another. Hence, more replication studies are warranted before any firm conclusions can be made.

Key words: Epidermal growth factor; Colorectal cancer; Vascular endothelial growth factor

INTRODUCTION

Tumorigenesis is initiated by multiple genetic and epigenetic alterations that confer growth and survival advantages to tumor cells. Unlimited cell proliferation and differentiation, as well as the inhibition of apoptosis will lead to the formation of hyperplastic growth, an early hallmark of cancer development. In the subsequent events of neoplastic progression, the further outgrowth of tumor cells is permitted by sustained angiogenesis, which not only supplies the oxygen and nutrients to the growing tumor cells, but also provides a potential route for the dissemination of tumor cells in cancer metastases (Baeriswyl and Christofori, 2009).

In humans, the epidermal growth factor receptor (EGFR) signaling pathway and its various downstream signal transduction pathways are tightly regulated and are responsible for several key cellular events including proliferation, differentiation, migration, and apoptosis. Epidermal growth factor (EGF) is one of the natural ligands of the EGFR, and the EGF/EGFR interaction was postulated as an important autocrine loop in conferring growth advantages to tumor cells (Gleave et al., 1993). It was reported that the genetic alterations and aberrant expression of various elements in these pathways impacted neoplastic transformation and progression via increased cell proliferation, prolonged survival, angiogenesis, and evasion of apoptosis (Mitsudomi and Yatabe, 2010). On the other hand, vascular endothelial growth factor (VEGF), which is the key regulator of both physiological and pathological angiogenesis in a plethora of angiogenic factors identified thus far, is commonly associated with poor cancer prognosis (Guba et al., 2004). Veikkola and Alitalo (1999) demonstrated that VEGF could stimulate endothelial proliferation, motility, and capillary morphogenesis *in vitro*, as well as regulate vascular permeability *in vivo*. Moreover, the suppression of tumor-induced angiogenesis and tumor growth via the inhibition of VEGF signaling further elucidated its role in carcinogenesis (Ferrara, 2002).

In this study, we focused on the genetic variants of both the *EGF* and *VEGF* genes, i.e.,

the *EGF* +61 A>G and *VEGF* +936 C>T polymorphisms, to investigate their roles in tumor biology since these low-penetrance, functional single nucleotide polymorphisms (SNPs) were postulated to modulate the individual cancer risk and angiogenic potential, suggesting that these variants might contribute to inter-individual differences in cancer susceptibility and severity.

MATERIAL AND METHODS

Study cohort

Our study cohort consisted of 130 colorectal cancer (CRC) patients and 212 healthy controls. The CRC patients had all been admitted to the University Malaya Medical Centre (UMMC) in Kuala Lumpur or to the Queen Elizabeth Hospital in Sabah, Malaysia, for surgical resection. All patients were newly diagnosed and manifested with sporadic CRC of different stages, ranging from stages I-IV. These recruited patients were aged between 40 and 90 years. Furthermore, age-matched control samples were obtained from healthy volunteers. The collection of blood samples was performed with written informed consent and the sampling procedures were approved by the Medical Ethics Committees of both institutions.

Genotyping of the *EGF* and *VEGF* polymorphisms

Genomic DNA was first isolated via a conventional DNA extraction method as reported previously (Puah et al., 2007; Chua et al., 2009, 2011). Subsequently, real-time polymerase chain reaction (PCR) with TaqMan chemistry was conducted to genotype both the *EGF* +61 A>G and *VEGF* +936 C>T polymorphisms. The PCR amplification was performed on the Applied Biosystems 7500 Fast Real-Time PCR System with the following PCR constituents: 5 µL 2X GTXpress Master Mix (Applied Biosystems, USA), 0.5 µL 20X TaqMan SNP Genotyping Assay (Applied Biosystems), 20 ng/µL DNA, and ddH_2O. The TaqMan SNP Genotyping Assays used in our study were pre-designed and made commercially available by Applied Biosystems: C_27031637_10 (*EGF* +61 A>G) and C_16198794_10 (*VEGF* +936 C>T). A universal thermal cycling protocol recommended by the manufacturer was used for the genotyping of the *EGF* and *VEGF* polymorphisms: initial holding step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s, and an annealing/extension step at 60°C for 30 s.

Statistical analysis

All genotyping data were analyzed by using the TaqMan Genotyper ver. 1.0.1 software (Applied Biosystems). The genotype frequencies for the *EGF* and *VEGF* polymorphisms were calculated in both the CRC patient and healthy control groups. The Fisher exact test was performed and the odds ratio was determined with 95% confidence interval via the SPSS ver. 20.0.0 software (IBM, USA).

RESULTS

All three genotypes in each of the *EGF* +61 A>G and *VEGF* +936 C>T polymorphisms were identified, and the G (69.2%) and C (86.0%) alleles were found with the highest

frequency in our population. The GG and CC genotypes of the respective *EGF* and *VEGF* variants were more frequent among the CRC patients, but the differences did not reach statistical significance (P > 0.05) (Table 1). Thus, neither the *EGF* +61 A>G or *VEGF* +936 C>T polymorphisms were associated to disease susceptibility in Malaysian CRC patients.

Table 1. Genotype frequency, P value and OR with 95%CI for both *EGF* and *VEGF* gene variants in CRC patient and healthy control groups.

Gene variant	Genotype frequency		P	OR (95%CI)
	CRC patient	Control		
EGF +61 A>G				
A/A	14 (10.8%)	13 (6.1%)	0.1486	1.8475 (0.8394-4.0662)
A/G	53 (40.8%)	104 (49.1%)	0.1471	0.7148 (0.4597-1.1115)
G/G	63 (48.4%)	95 (44.8%)	0.5765	1.1581 (0.7476-1.7941)
VEGF +936 C>T				
C/C	99 (76.2%)	151 (71.2%)	0.3794	1.2901 (0.7816-2.1294)
C/T	31 (23.8%)	57 (26.9%)	0.6106	0.8515 (0.5139-1.4108)
T/T	0	4 (1.9%)	0.3018	-

DISCUSSION

Besides oncogenes and tumor suppressor genes, the family of growth factors also plays an important role in malignant transformation and progression. In fact, the relationship between growth factors (i.e., EGF, platelet-derived growth factor, transforming growth factors, insulin-like growth factors, fibroblast growth factors, VEGF, etc.) and cancer has long been well established (Goustin et al., 1986). For instance, aberrant expression of both EGF and EGFR leading to malignant phenotypes of tumor cells has been reported in several cancer types, i.e., cancers of the lung, colon, head and neck, breast, and ovary (Krasinskas, 2011). Furthermore, the increased expression of VEGF was also demonstrated in several human malignancies such as breast cancer and malignant mesothelioma (Strizzi et al., 2001; Toi et al., 2001).

EGF was one of the first growth factors to be identified in humans (Cohen, 1983). The binding of EGF to EGFR activates the Ras/Raf/mitogen-activated protein kinase and phosphatidylinositol-3-kinase pathways, which are responsible for the proliferation, differentiation, and tumorigenesis of epithelial tissues (Jorissen et al., 2003). We here focused on a functional polymorphism in the 5'-untranslated region of the *EGF* gene, +61 A>G (rs4444903), owing to its demonstrated role in influencing gene transcription, which consequently modulates the serum level of EGF. The substitution of guanine (G) for adenine (A) at the rs4444903 locus was shown to cause a significant increase in EGF expression in cultured peripheral blood mononuclear cells (Shahbazi et al., 2002). The modulation of EGF serum levels by this promoter variant, i.e., G allele carriers had higher levels of EGF production, was further supported by other studies on human carcinomas (Lanuti et al., 2008; Tanabe et al., 2008). To date, the +61 A>G variant is the only functionally identified SNP in the *EGF* gene, and numerous epidemiological studies have been performed in an attempt to establish its potential association to cancer susceptibility. However, the findings obtained thus far are inconsistent and no concrete conclusion about this association can be drawn at present (Zhang et al., 2010).

It is noteworthy that the observed distribution of the *EGF* +61 A>G polymorphism among the Malaysian population was similar to those reported in other Asian cohorts. The GG genotype was found at a higher frequency (44.8%) compared to the homozygous A genotype

(6.1%) among the control subjects in this study. Previous studies demonstrated the existence of ethnic heterogeneity in the distribution of the *EGF* +61 A>G polymorphism, especially between Caucasians and Asians (Zhang et al., 2010). The homozygous G genotype was found at a higher frequency compared to the homozygous A genotype among Asian populations, i.e., in Japanese (47.8%), Korean (51.5%), and Chinese (47.6%) populations (Goto et al., 2005; Kang et al., 2007; Gao et al., 2008). However, in Caucasian populations, the AA genotype was found in higher frequency than its GG counterpart (Shahbazi et al., 2002; Vauleon et al., 2007; Lanuti et al., 2008). According to two different meta-analyses on the association between the *EGF* promoter variant and cancer risk, the *EGF* +61 A>G polymorphism was proposed to be correlated with an increased risk of gastric, esophageal, and colorectal cancers, as well as glioma (Zhang et al., 2010; Piao et al., 2013). The GG genotype was reported to be significantly associated to CRC susceptibility among Caucasians (Wu et al., 2009). In addition, the homozygous state of the G allele also confers susceptibility to gallbladder cancer and malignant melanoma (Shahbazi et al., 2002; Vishnoi et al., 2008). Nevertheless, other studies found contradictory findings in which the *EGF* +61 A>G polymorphism was not a significant risk factor for colorectal and gastric cancers or glioblastoma (Goto et al., 2005; Vauleon et al., 2007; Yu and Weng, 2011). In our study, there was no significant difference in the genotype frequencies between the CRC patients and healthy controls. Thus, the *EGF* +61 A>G polymorphism does not appear to be involved in the genetic predisposition to CRC in this Malaysian population. This finding supports the results of another epidemiological study on the *EGF* gene and CRC in an Iranian population (Daraei et al., 2012).

The promotion and sustainment of tumor cell survival are crucial factors for cancer initiation, whereas angiogenesis plays a predominant role in tumor invasion and metastasis. In carcinogenesis, the fine-tuned balance between the pro- and anti-angiogenic factors is disrupted and leads to an angiogenic switch. The pro-angiogenic activities are favored in malignancy for the development of new blood capillaries from the endothelium of the pre-existing vasculature (Ribatti et al., 2007). Of all factors involved in these processes, VEGF is the key regulator in both vasculogenesis and angiogenesis through its specific binding to the tyrosine kinase receptor, VEGF receptor-2, in endothelial cells. The *VEGF* gene is located on chromosome 6p21.3, and at least 30 SNPs have been identified thus far (Dassoulas et al., 2009). In our study, we focused on the +936 C>T polymorphism (rs3025039) in the 3'-untranslated region owing to its relationship to the circulating plasma level and tumor expression level of VEGF. Renner et al. (2000) demonstrated that the C>T substitution was correlated to a lower plasma level of VEGF due to the loss of the potential binding site for the transcription factor AP-4. As reported previously in a meta-analysis, the +936 T allele was associated with an increased risk of oral cancer. The T allele was proposed to exert an augmentative effect on cancer risk among Asian and European populations, whereas it played a protective role in cancer predisposition in an African population (Xu et al., 2010). In fact, several conflicting findings were reported with respect to the role of the T allele in mediating cancer risk; it was associated with an increased risk for gastric and oral cancers, but with a decreased risk of developing breast cancer (Krippl et al., 2003; Yapijakis et al., 2007; Bae et al., 2008).

Unlike *EGF* +61 A>G, the genotype distribution of the *VEGF* +936 C>T polymorphism is similar across different populations, with CC homozygotes being the most frequent, with frequencies of 70.4, 66.4, 73.8, and 72.1% in Polish, Chinese, Korean, and Austrian populations, respectively (Jin et al., 2005; Kataoka et al., 2006; Bae et al., 2008; Hofmann et al.,

2008). In our study, the CC genotype was found at the highest frequency of 71.2%. However, none of the *VEGF* genotypes was significantly associated with susceptibility to CRC. Our findings were similar to those of previous studies conducted in other populations in which no correlation between the *VEGF* +936 C>T variant and CRC susceptibility was observed (Hofmann et al., 2008; Dassoulas et al., 2009; Wu et al., 2009). Nonetheless, the TT genotype was shown to correlate with the advanced stage of CRC, a higher serum level of CA19-9, a higher histological grade of CRC tumors, and poorer patient prognosis (Chae et al., 2008). Furthermore, Nakasaki et al., (2002) demonstrated that the expression of VEGF varied significantly across different stages of CRC progression, and was correlated with the metastasis and tumor vascularity of CRC neoplasms.

CONCLUSION

None of the *EGF* +61 A>G or *VEGF* +936 C>T polymorphisms investigated in this study was associated to a genetic predisposition to CRC in the Malaysian population. The genetic distribution of the *EGF* +61 A>G variant shows ethnic heterogeneity and exerts different impacts on CRC risk in different populations. On the other hand, several genome-wide association studies also failed to demonstrate an association between the *VEGF* +936 C>T variant and the risks of breast, colon, and prostate cancers. Because the complex VEGF-mediated signaling network is believed to be governed by multiple factors, the final effect of this angiogenic pathway on carcinogenesis is likely to be influenced by interactions between various signaling molecules and receptors rather than by VEGF alone.

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