

Allele Frequencies of the Epidermal Growth Factor Receptors Polymorphism R521K in Colorectal Cancer Patients and Healthy Subjects Indicate a Risk-Reducing Effect of K521 in Syrian Population

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Abstract

Background: Colorectal cancer contributes heavily to cancer morbidity and mortality worldwide. Numerous therapies are currently in use, including monoclonal antibodies against cellular components involved in tumorigenesis such as epidermal growth factor receptors (EGFRs). Studies showed the polymorphism [R521K] GÀA in the EGFR gene to be involved in both colorectal cancer susceptibility and clinical response to therapeutics (e.g., Cetuximab). **Aim:** We aimed at uncovering allele frequencies of this polymorphism among Syrian colorectal cancer patients and healthy individuals. **Materials and Methods:** Forty-seven patients with colorectal cancer were included in a case–control study along with 48 healthy subjects, all native Syrians. Individuals were genotyped using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and results were statistically analyzed to elucidate significant differences between the two groups. **Results:** Allele frequencies were 40.4% (G/G), 57.4% (G/A) and 2.1% (A/A) in colorectal cancer patients and 41.6% (G/G), 43.7% (G/A) and 14.5% (A/A) in healthy subjects. The A/A genotype was significantly lower in colorectal cancer patients than in the control group. **Conclusions:** Homozygosity for the A allele is linked to reducing the risk of developing colorectal cancer in Syrian patients. The lower prevalence of (A/A) locally may predict sub-optimal rates of clinical response to Cetuximab compared with populations with higher frequencies of the A allele. Larger scale investigations are needed for a stronger conclusion.

Keywords: Colorectal cancer, Epidermal growth factor receptor, Polymorphism, R521K, Syrian

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Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, with high metastasis and poor survival rates. CRC clearly follows the multistage

model; initially affecting normal mucosa and finishing up as an invasive adenocarcinoma, with transitional stages including dysplastic lesions, adenoma and *in situ* adenocarcinoma.^[1-4] This sequence of events happens as a result of the accumulation of genetic aberrations, which ultimately leads to dysfunctional changes in cell proliferation, differentiation and apoptosis.^[1,5,6] Eventually, such imbalance between the rates of cell growth and apoptosis results in the development and progression of CRC.

The genomic picture of CRCs is highly complex, with up to 70 mutations in well-established cancer genes being found to be involved in tumor initiation and

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progression.^[7] One of the most extensively studied genes in various cancers is the one encoding epidermal growth factor receptor (EGFR). EGFR, a transmembrane glycoprotein, is a member of the human epidermal growth factor receptor (HER/Erbb) family of receptor tyrosine kinases. The timely activation of certain tyrosine kinases initiates transduction cascades of several signaling pathways, including ras/raf/MAPK (Mitogen-activated protein kinases) and phosphatidylinositol-3-kinase, which are involved in gene expression, cell growth, cell proliferation, angiogenesis and apoptosis.^[8-12] The central role of EGFR signaling in cell growth and survival implies that alterations in the function of EGFR may have a substantial impact in the development and progression of cancer.

A considerable mass of published work provided evidence for the role of EGFR abnormalities in several types of cancer including those of the lung, colon, head and neck, pancreas, breast, ovary, bladder, kidney and gliomas.^[13-20] In fact, the activity of EGFR was shown to be elevated in most solid tumors. In response to this data, attention was directed toward EGFR variants, and indeed it was shown that many EGFR polymorphisms play a significant role in the development and prognosis of CRC.^[21-25] One of these is R521K, or R497K according to an older nomenclature, which is a functional polymorphism in EGFR that arose from a G-to-A transition, resulting in an Arg to Lys substitution. Substituting arginine by lysine in codon 521 on the border between extracellular subdomains III and IV of EGFR causes a reduction in the activity of the receptor. This may be attributed to attenuating ligand binding and ligand-induced EGFR signaling.^[26] Moreover, it has been reported that the variant R521K can predict a better response to the anticancer drug Cetuximab in combination with irinotecan.^[27]

This study aims to investigate allele frequencies of the functional polymorphism R521K in *EGFR* in order to find potential associations between these frequencies and CRC in the Syrian population. This regionally unprecedented screening is an important beginning to many feasibility studies focusing on introducing anti-EGFR antibodies (e.g., Cetuximab) into therapeutic protocols for treating Syrian CRC patients. This assessment may assist local health authorities in setting up their policies with regard to procuring Cetuximab and similar medications.

Materials and Methods

Subjects

This case-control study was conducted on a group of 47 operated-on patients with confirmed diagnosis of colon

cancer. The control group consisted of 48 healthy subjects with no history of cancer. Patients were recruited from the University Hospital in Aleppo, Syria. All subjects were native Syrians and of the same ethnicity (Arabs). Blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes and anonymously coded and stored. Informed consent was obtained from patients and healthy subjects. The study was approved by the Ethical Committee at the University of Aleppo.

DNA isolation and PCR

Genomic DNA was extracted from 200 µl of venous blood using a spin column format kit (Fermentase, Lithuania). The isolation of DNA was carried out according to the manufacturer's instructions.

The R521K (G < A) polymorphism of the EGFR gene was genotyped using a previously described PCR-RFLP method using the restriction enzyme MvaI.^[1] The PCR reaction was carried out in a total volume of 20 µl, containing 4 µl of genomic DNA as template, 0.4 µM of each primer (synthesized by VBC-Biotech, Austria), 2 µM of PCR buffer, 0.4 µM of each dNTP, 2 µM of MgCl₂, 1X Taq buffer and 1 unit of Taq DNA Polymerase (Fermentase, Lithuania). PCR amplification was carried out in a MasterCycler® thermal cycler (Eppendorf, Germany), with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 10 s, and a final extension at 72°C for 5 min.

The sequences of the forward and reverse primers were: 5'-TGCTGTGACCCACTCTGTCT-3' and 5'-CCAGAAGGTTGCACTTGTCC-3', respectively.

The PCR product (155 bp) was digested with MvaI restriction enzyme (FastDigest® Fermentase) at 37°C for 10 min. This restriction enzyme recognizes the sequence CC/WGG. The amplicon with (G) is cleaved twice by the enzyme, rendering three fragments (38, 50 and 67 bp), whereas the A variant is cleaved only once (38 and 117 bp).

Digestion products were separated by electrophoresis on 2% Nusieve ethidium bromide-stained agarose gels (TopVision Agarose, Fermentas, Lithuania) and visualized under UV light. Analyses by sex and age were skipped due to the small sample size.

Statistical analysis

Standard contingency table analysis and Chi-square test were used to assess the association of the genotype and allele frequencies for the EGFR R521K polymorphism with CRC. Risk value to CRC was estimated by calculating the odds ratios with 95% confidence intervals. *P* values < 0.05 were considered statistically significant.

Results

The R521K polymorphism was genotyped in CRC patients and healthy subjects. The results are summarized in Table 1, which showed that the frequencies of the G/G, G/A and A/A genotypes in healthy subjects were 41.6% ($n = 20$), 43.7% ($n = 21$), and 14.5% ($n = 7$), respectively. In the colorectal patients' group, the frequencies of the G/G, G/A and A/A genotypes were 40.4% ($n = 19$), 57.4% ($n = 27$) and 2.1% ($n = 1$). Data analysis indicates that the A/A genotype frequency is significantly different between patient and control groups ($\chi^2 = 4.05$, $df = 1$, $P = 0.04$). The frequencies of the G and A alleles were 63.5% ($n = 61$) and 36.4% ($n = 35$), respectively, in the control group and 69.1% ($n = 65$) and 30.85% ($n = 29$), respectively, in CRC patients. No significant difference in the allele frequency was observed between the two groups ($\chi^2 = 0.14$, $df = 1$, $P = 0.7$, and $\chi^2 = 0.33$, $df = 1$, $P = 0.56$ for the G and A alleles, respectively).

Discussion

The EGFR polymorphic variant (142285 G > A) results in an arginine (R)/lysine (K) substitution in codon 521 in the extracellular domain of EGFR. This substitution is associated with attenuation in the affinity and ligand binding to the EGFR, which leads to reduced activation of downstream tyrosine kinase activity in downstream mitogenic pathways.^[26,28]

In line with these observations, the R521K polymorphism is speculated to be linked with less susceptibility to cancer, and indeed several studies confirmed better prognostic features in more than one type of cancer.^[25,29-32] In CRC, there are a number of studies that highlighted the association between G/A or A/A and decreased tumor recurrence, lower probability of subsequent metastasis and longer survival in post-surgery CRC patients. The

R521K polymorphism of EGFR was also associated with better response and survival in CRC patients treated with 5-FU and oxaliplatin-based chemotherapy, Cetuximab as single agent or in combination with irinotecan and with FOLFOX-4.^[22,25,27,32-36]

However, only one study showed an association of this polymorphism with a decreased risk of CRC.^[37]

The aim of the current study was to reveal local allele frequencies of the non-synonymous EGFR variant R521K. Such data is critical to evaluate the worth of using this polymorphism as a biomarker for susceptibility to CRC and to predict the clinical response to drugs such as Cetuximab in the Syrian population. The latter point is highly relevant to address the feasibility of using the new anti-EGFR drug Cetuximab in the treatment of Syrian CRC patients. This can be based on the findings that certain alleles of this polymorphism correlate with a higher response rate and a favorable prognosis in Cetuximab-treated CRC patients.

In this study, we assessed R521K polymorphism genotype frequencies in Syrian colorectal patients and compared these frequencies with those from a representative control sample of the Syrian population.

Importantly, our results showed that R521K genotype frequencies in the CRC cohort were significantly different from those observed in the control cohort. Our results are consistent with the findings of an Italian study that suggested a negative correlation between R521K and susceptibility to CRC.^[37] However, our data differed from the latter study because it showed that only A/A genotype was significantly different between the two groups. This discrepancy could be explained by the ethnic differences that may have an impact on susceptibility to cancer. Our results showed for the first time that allelic distribution of this polymorphism in the Syrian population was distinct, and remarkably different, from other ethnic groups [Table 2].

Compared with other populations, the prevalence of A/A genotype was remarkably higher in healthy Syrian subjects than in Europeans, African Americans and Sub-Sahara Africans, but significantly lower than that of Asians [Table 2] (Single Nucleotide Polymorphism (SNP) database, ncbi.nlm.nih.gov). It is well established that the ethnic differences may have a considerable influence on susceptibility to cancer as well as on clinical response profiles to chemotherapy.^[38-41] Based on the findings that this polymorphism influences the anticancer activity of Cetuximab, possibly by enhancing the anticancer effect of Cetuximab, our results and previous findings in different ethnic groups suggest that Syrian patients might have poorer outcome to Cetuximab-based treatment than

Table 1: R521K genotype and allele frequencies in patients with colorectal Syrian cancer patients and in healthy subjects

Group	Control (%)	Patients (%)	P value
Total number	48	47	
G/G	20 (41.6)	19 (40.4)	0.94
G/A	21 (43.7)	27 (57.4)	0.44
A/A	7 (14.5)	1 (2.1)	0.04
G	61 (63.5)	65 (69.1)	0.71
A	35 (36.4)	29 (30.85)	0.57

A/A: Pertaining to genotype A/A of the G→A polymorphism in EGFR;
G/A: Pertaining to genotype G/A of the G→A polymorphism in EGFR;
G/G: Pertaining to genotype G/G of the G→A polymorphism in EGFR;
G: Pertaining to the G allele of the G→A polymorphism in EGFR in the gene pool;
A: Pertaining to the A allele of the G→A polymorphism in EGFR in the gene pool

Table 2: R521K genotype distribution in different ethnic groups

Ethnicity	A/A (%)	G/A (%)	G/G (%)
Europeans	0	50	50
African Americans	0	21.7	78.3
Asian	20.8	58.3	20.8
global	12.0	33.7	54.2
Asian	22.5	47.5	30.0
Asian	32.9	57.6	9.4
Sub-sahara African	0	10.7	89.3
Europeans	8.1	40.5	51.4
Chinese	27.6	51	21.4
Syrians	14.5	43.7	41.6

A/A %: Percentage of genotype A/A of the G→A polymorphism in EGFR;
 G/A %: Percentage of genotype G/A of the G→A polymorphism in EGFR;
 G/G %: Percentage of genotype G/G of the G→A polymorphism in EGFR;
 Source: SNP (Single nucleotide polymorphism), database www.ncbi.nlm.nih.gov

Asian CRC patients. However, more cross-ethnic studies on a larger scale are required to confirm this line of reasoning.

In conclusion, our study demonstrates for the first time that one genotypic variant of R521K polymorphism, namely the A/A, confers a protective advantage in relation to CRC in the Syrian population. Indeed, our data confirmed the negative correlation between the A/A variant and susceptibility to CRC. Finally, considering only the R521K polymorphism, especially the very low frequency of A/A in Syrian patients as a predicting factor of Cetuximab treatment, it appears that the use of Cetuximab might not produce a very good outcome in Syrian CRC patients. This presents a weak case for introducing Cetuximab by health authorities in the Syrian Arab Republic and studies with larger sample sizes are called for to reach the best possible conclusion in this regard.

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