

Analysis of the polymorphisms EGFR-R521K and ERBB2-I655V in Mexican patients with gastric cancer and premalignant gastric lesions

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ABSTRACT

The proto-oncogenes epidermal growth factor receptor (EGFR) and erythroblastic leukemia viral oncogene homolog 2 (ERBB2), are involved in the development of diverse malignant tumors, including gastric cancer. We analyzed the association of SNPs EGFR-R521K and ERBB2-I655V with gastric cancer and premalignant gastric lesions in Mexican patients. Through restriction fragment length polymorphisms, we analyze both SNPs in the DNA from 155 patients with gastric cancer and premalignant gastric lesions, 121 controls, and 103 people from the Mexican general population. The frequencies of both SNPs did not differ significantly between any of the groups (χ^2 p=NS); Odds ratio analysis showed that the alleles EGFR-521K and ERBB2-655V were not related to gastric cancer or premalignant gastric lesions in the Mexican population. Our data suggest that the EGFR-R521K and ERBB2-I655V polymorphisms are not suitable as markers for identifying individuals with a higher risk for developing gastric cancer in our population.

Key words. Gastric cancer. Gastritis. Intestinal metaplasia. EGFR. HER-2.

Análisis de los polimorfismos EGFR-R521K y ERBB2-I655V en pacientes mexicanos con cáncer gástrico y lesiones gástricas premalignas

RESUMEN

Los proto-oncogenes epidermal growth factor receptor (EGFR) y erythroblastic leukemia viral oncogene homolog 2 (ERBB2), se encuentran involucrados en el desarrollo de diversos tumores malignos, incluyendo cáncer gástrico. En este trabajo se analiza la asociación de los SNPs EGFR-R521K y ERBB2-I655V con cáncer gástrico y lesiones gástricas premalignas en pacientes mexicanos. Mediante análisis de polimorfismos en la longitud de fragmentos de restricción, se analizaron ambos SNPs en el ADN de 155 pacientes con cáncer gástrico y lesiones gástricas premalignas, 121 controles, y 103 sujetos de población general. Las frecuencias de ambos SNPs no difirieron significativamente entre los grupos (χ^2 p = NS); el análisis de razón de momios mostró que los alelos EGFR-521K y ERBB2-655V no están relacionados con cáncer gástrico o lesiones gástricas premalignas en la población mexicana. Los datos sugieren que los polimorfismos EGFR-R521K y ERBB2-I655V no pueden considerarse como marcadores útiles en la identificación de sujetos con riesgo de desarrollar cáncer gástrico en nuestra población.

Palabras clave. Cáncer gástrico. Gastritis. Metaplasia intestinal. EGFR. HER-2.

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer worldwide, with 934 000 cases per year.^{1,2} In Mexico, in 2008, stomach tumors had an incidence of 9.4 per 100,000 in men and 6.7 per 100,000 in women.³ Stomach cancer is the second most frequent type of cancer in terms of mortality in the world.²

The family of tyrosine kinase receptors human epidermal receptor (HER) includes HER-1, also called epidermal growth factor receptor (EGFR), HER-2, HER-3, and HER-4.⁴ These receptors are important regulators of cellular functions such as cell growth and differentiation.⁵ The binding of a ligand to the receptor leads to homo- or heterodimerization of these receptors, and the consequent autophosphorylation of their cytoplasmic residues, which allows the coupling of proteins involved in signal transduction.⁶

The HER receptors are involved in the pathogenesis of various cancers, and their overexpression is associated with poor prognosis.⁴ For example, some mutations located in exons 18-24 of the EGFR gene, which encoded for the transmembrane domain of HER-1, participates in the development of non-small cell lung cancer.⁷ HER-1 is overexpressed principally in glioblastoma, lung, colorectal, and head and neck cancers; in gastric cancer, the EGFR expression has been linked to advanced clinical stage and the presence of lymph node metastasis.⁸ The amplification and/or overexpression of HER-2 (Human Epidermal Growth Factor Receptor 2) also known as Neu or ERBB-2, have been implicated in the development of breast, ovary, bladder, pancreatic, prostate, and gastric carcinoma, and other solid tumors.^{9,10}

Several single nucleotide polymorphisms (SNPs) are associated with different types of cancer. For example, R521K (rs2227983) on the EGFR gene is associated with an improved prognosis in colorectal cancer,^{11,12} this polymorphism has not been studied in gastric cancer; and the SNP I655V (rs1136201) on the erythroblastic leukemia viral oncogene homolog 2 gene (ERBB2) has been studied in breast^{13,14} and gastric cancer with positive results of association.^{15,16}

In this study, we analyzed the association between two SNPs, EGFR-R521K and ERBB2-I655V, and GC and premalignant gastric lesions in Mexican subjects.

MATERIALS AND METHODS

Study population

We analyzed the genomic DNA from 155 Mexican patients with different gastric lesions determined by

histology, which were divided into two groups: gastric cancer (n = 73, 95.8% were classified as adenocarcinomas and 4.2% as lymphomas), and premalignant gastric lesions (PGL) [n = 82, 92.7% were classified as intestinal metaplasia (IM) and 7.3% as atrophic gastritis (AG)].

A control group (without premalignant lesions) was also studied, this included DNA samples of 121 subjects with nonatrophic chronic gastritis (NAG); besides, we studied DNA samples from 103 healthy blood donors, unrelated and older than 18 years from western of Mexico, which partially represents the Mexican general population (MGP). All participants signed an informed consent form. The study was approved by a Local Committee on Research in Health. The DNA was extracted from fresh blood by the salting-out method.¹⁷

R521K (g.147531 G-A) polymorphism

A fragment of 158 base pairs (bp) was amplified by polymerase chain reaction (PCR) using the following primers: forward, 5'-TGT TGT GAC CCA CTC TGT CTC CG-3', and reverse, 5'-CCT CCA GAA GGT TGC ACT TGT CC-3'.¹⁸ The reaction was performed with 200 ng of genomic DNA, 5 pM of each primer, 0.5 U of Taq polymerase, 0.017 mM of MgCl₂, 1 x PCR buffer, and 2 μM of deoxynucleotide triphosphates (dNTPs). The program comprised initial denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min, and the reaction was completed at 4 °C for 10 min. The PCR products were digested with 1.25 U of Bst-NI enzyme (New England BioLabs, Ipswich, MA) at 60 °C for 2 h. The resulting fragments were visualized by electrophoresis in 6% polyacrylamide gels and were stained with silver nitrate.¹⁹ The expected restriction fragments were:

- G/G = 38 bp + 50 bp + 70 bp.
- G/A = 38 bp + 50 bp + 70 bp + 120 bp.
- A/A = 38 bp + 120 bp.

I655V (g.40196 A-G) polymorphism

The PCR reaction was performed with primers previously described: forward, 5'-AGA GCG CCA GCC CTC TGA CGT CCA T-3', and reverse, 5'-TCC GTT TCC TGC AGC AGT CTC CGC A-3'. The reaction was performed with 200 ng of genomic DNA, 0.02 pM of each primer, 0.5 U of Taq polymerase, 0.102 mM of MgCl₂, 1 x PCR buffer, and 2 μM of

dNTPs. The amplified fragment of 148 bp was subjected to enzymatic digestion using 2 U of BsmAI enzyme (New England BioLabs) at 55°C for 1 h. The resulting fragments were visualized by electrophoresis in 6% polyacrylamide gels and were stained with silver nitrate. The expected restriction fragments were:

- A/A = 148 bp.
- A/G = 32 bp + 116 bp + 148 bp.
- G/G = 32 bp + 116 bp.

Statistical analysis

The genotype and allele frequencies were calculated for each group and are shown in table 2. The Hardy-Weinberg equilibrium (HWE) test was performed in the general population group for both SNPs using the Epi Info™ program version 3.3. The association of the EGFR-R521K and ERBB2-I655V polymorphisms, with GC or premalignant gastric lesions (PGL) was calculated, and was expressed as odds ratios (ORs) with 95% confidence intervals.

RESULTS

The age means observed in our groups were heterogeneous (Table 1). The GC group was the oldest, followed by the IM, and AG groups (analysis of variance $p = 0.1226$). The age of the combined GC and PGL groups also differed from that of the NAG control group ($p < 0.001$). The sex distribution also differed between the groups studied. In the GC and the AG groups, males were affected more; the male-to-female ratios were 2.3:1 and 2:1, respectively, although the AG group was very small ($n = 6$). The ratios were 0.62:1 in the IM group, and 1.4:1 in the MGP group. By contrast, women were most affected in the NAG group (male-to-female ratio, 0.37:1).

The distribution of genotype frequencies of the EGFR-R521K and ERBB2-I655V SNPs did not differ significantly between the GC, PGL and control group (chi-square test $p = 0.815$ and $p = 0.965$, respectively). The allele frequencies of EGFR-R521K and ERBB2-I655V, also were similar in the GC, PGL, and NAG groups (chi-square test $p = 0.804$, and $p = 0.957$ respectively) (Table 2).

Table 1. Characteristics of the groups studied.

	AG n = 6	GC n = 73	IM n = 76	NAG n = 121	MGP n = 103
Age					
Mean	51	61.1	58	48.9	32.6
± SD	18.4	14.1	12.5	13.4	9.9
Range	31-82	29-86	31-80	20-85	18-61
Sex					
Male n (%)	66.7	69.9	38.2	27.3	58.3
Female n (%)	33.3	30.1	61.8	72.7	41.7

AG: atrophic gastritis. GC: gastric cancer. IM: intestinal metaplasia. NAG: nonatrophic gastritis. MGP: Mexican general population.

Table 2. Genotypic and allele frequencies of the R521K and I655V SNPs.

		GC	PGL	NAG	MGP
EGFR-R521K (G-A)	GG n (%)	31 (43.1)	36 (48.0)	42 (42.0)	49 (49.0)
	GA n (%)	33 (45.8)	28 (37.3)	43 (43.0)	43 (43.0)
	AA n (%)	8 (11.1)	11 (14.7)	15 (15.0)	8 (8.0)
	G %	66	66.7	63.5	70.5
	A %	34	33.3	36.5	29.5
ERBB2-I655V (A-G)	AA n (%)	49 (68.1)	55 (67.1)	84 (69.4)	76 (73.8)
	AG n (%)	20 (27.8)	25 (30.5)	33 (27.3)	25 (24.3)
	GG n (%)	3 (4.2)	2 (2.4)	4 (3.3)	2 (1.9)
	A %	81.9	82.3	83.1	85.9
	G %	18.1	17.7	16.9	14.1

GC: gastric cancer. PGL: premalignant gastric lesions. NAG: nonatrophic gastritis. MGP: Mexican general population.

In the Mexican general population group, the allele frequencies observed were 29.5% and 14.1% for EGFR-521K (A) and ERBB2-655V (G), respectively (Table 2). The HWE was observed for both SNP's, EGFR-R521K ($p = 0.944$) and ERBB2-I655V ($p = 0.999$). We compare the allele frequencies of EGFR-R521K observed in MGP group with that reported for seven populations in SNP database.²⁰ The distribution of allele 521K (A) did not show significant differences with Americans with European ancestry (26.7% $p = 0.747$), Italians (24.4% $p = 0.459$), Indians (35.1% $p = 0.315$) and Mexicans (29% $p = 0.990$). In the other hand, the allelic distribution of 655V (G) also was compared with the 56 populations reported in ALFRED²¹ and SNP databases,²² we not observed significant differences with the most Asian and East Asian populations (8-23.8% $p = NS$), Oceanics (4.4-17.6% $p = NS$); some European populations like Adygei (17.6%), Chuvash (22%), Jews Ashkenasi (14.2%), and Italians (12.5%) ($p = NS$); North America populations like Cheyennes (12.5%), Mayas (12.2) and Mexicans (13.3%) ($p = NS$); and South American populations as Karitiana (9.3%) and Quechúa (8.7%) ($p = NS$).^{21,22} In our study the frequencies of 521K (A) and 655V (G) alleles were similar to European and Asian populations, this is consistent with that reported in studies of population markers in Mexican population,²³ which is explainable because is known that the genetic composition of the Mexican population is constituted principally by Asian, Caucasian and a minority of African genes.^{24,25}

We used OR analysis to compare the GC group and the NAG group as controls (Table 3). We also compare the PGL and GC groups combined as one group with the NAG group. In both comparisons, the EGFR-R521K and ERBB2-I655V alleles or genotypes were not associated with GC alone or with AG, IM, and GC combined into one group. The ORs were not significant in all cases ($p > 0.05$) (Table 3).

DISCUSSION

Gastric carcinogenesis occurs most frequently in males, with a ratio of males to females of 2:1, and in people > 60 years of age. In our study the GC group was the oldest, in according to previously reported; males were more affected in the GC and AG groups. Although the reason for the sex difference is unclear, the possible protective role of estrogens has been proposed.^{26,27}

Table 3. Association analysis of the EGFR-R521K and ERBB2-I655V polymorphisms with gastric cancer and premalignant gastric lesions.

	EGFR-R521K (G-A)				ERBB2-I655V (A-G)			
	GA/AA vs. GG	AA vs. GG/GA	GA vs. GG/AA	GA/GG vs. AA	GG vs. AA/AG	AG vs. AA/GG	GG vs. AA/AG	AG vs. AA/GG
GC	41	8	33	23	3	20	3	20
Controls (NAG)	58	15	43	37	4	33	4	33
OR (95% CI) p	0.957 (0.52-1.77) 0.890	0.708 (0.28-1.77) 0.460	1.121 (0.61-2.06) 0.712	1.065 (0.57-1.99) 0.842	1.271 (0.26-5.65) 0.757	1.025 (0.53-1.97) 0.939	1.271 (0.26-5.65) 0.757	1.025 (0.53-1.97) 0.939
GC and PGL	80	19	61	50	5	45	5	45
OR (95% CI) p	0.865 (0.52-1.44) 0.578	0.841 (0.41-1.74) 0.642	0.940 (0.56-1.57) 0.814	1.091 (0.65-1.82) 0.738	0.981 (0.26-3.73) 0.978	1.100 (0.65-1.87) 0.722	0.981 (0.26-3.73) 0.978	1.100 (0.65-1.87) 0.722

GC: gastric cancer. NAG: no atrophic gastritis. PGL: premalignant gastric lesions. OR: odds ratio. CI: confidence interval.

GC is closely related to infection with *Helicobacter pylori*, which has been classified as a human carcinogen by the International Agency for Research on Cancer. The US Centers for Disease Control and Prevention estimate that two-thirds of the world population harbors the bacterium, but not all develop GC.²⁸ Generally, the infection is a long-term process, and the progression from normal gastric tissue to cancer takes several decades and has intermediate stages of chronic gastritis, gastric atrophy, and incomplete intestinal metaplasia and dysplasia. In many cases, these precursor lesions are asymptomatic and are not diagnosed until GC has developed to an advanced stage.^{2,29} In the subjects here studied we did not analyze the *Helicobacter pylori* infection; however this aspect should be considered in future studies.

In our study, neither allele EGFR-R521K nor ERBB2-I655V was associated with GC or premalignant gastric lesions appearing as AG, or IM. To our knowledge, no studies have reported an association of the SNP EGFR-R521K with GC or PGL; however, it is known that the allele 521K has attenuated function in ligand binding, growth stimulation, tyrosine kinase activation, and induction of the proto-oncogenes *myc*, *fos*, and *jun*.³⁰

On the other hand, has been proposed that the wild type allele (I655) of the ERBB2-I655V polymorphism could destabilize the formation of receptor active dimers, resulting in a reduced tyrosine kinase activity,³¹ studies have searched an association of this polymorphism with breast cancer have showed controversial results,¹⁴ the Frank, *et al.* study failed in found an association of the SNP I655V with breast cancer, however observed an adjacent polymorphism (I654V) associated to breast cancer and they hypothesize that the presence of two consecutive valine residues would stabilize the formation of active ERBB2 dimers, thus increasing autophosphorylation, tyrosine kinase activity and cell transformation, therefore others SNPs can be influence the function of I655V, and the controversial results observed in different populations could be explained by this circumstances.³²

Previously reported results with regards to ERBB2-I655V polymorphism and gastric cancer are discordant with our results; a study by Kuraoka, *et al.*, of 212 GC Japanese patients and 287 controls without cancer, selected by epigastralgia or appetite loss, reported an increased OR of the Val/Val genotype of 3.25 compared with the Ile/Ile genotype and that the heterozygous Ile/Val genotype had an intermediate OR of 1.97.¹⁵ A study by Satiroglu-Tufan, *et*

al. included 25 GC Turkish patients and 18 controls with chronic gastritis, and the genotype heterozygote (Ile/Val) was related to GC ($p = 0.04$).¹⁶

The differences on these polymorphisms between the current study and these studies^{15,16} may result of the study design. We consider that in studies of association in complex diseases, like cancer, is very important to analyze a larger sample size, as well as to perform a good selection of cases and controls paired by age and sex, with the aim to obtain a greatest statistical power.

For the other hand, the strength of the association also may depend on the studied population, so that major studies are required with various ethnic groups and with different genetic profiles. Thereby, studies of the association of EGFR-R521K and ERBB2-I655V polymorphisms with GC and PGL, requires additional research.

In conclusion, our data suggest that the EGFR-R521K and ERBB2-I655V polymorphisms, studied with our methodology, are not suitable as markers for identifying individuals with a higher risk for developing GC in the Mexican population. Furthermore, the genetic background of each population is important and should be considered before using SNPs as markers in estimate the risk of diseases or in applications such as pharmacogenomics.

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