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Research Article

Genetic Polymorphisms in the EGFR (R521K) and Estrogen Receptor (T594T) Genes, EGFR and ErbB-2 Protein Expression, and Breast Cancer Risk in Tunisia

Imen Kallel,[1] Maha Rebai,[1] Abdelmajid Khabir,[2] Nadir R. Farid,[3] and Ahmed Rebaï[1]

[1] *Bioinformatics and Signalling Unit, Centre of Biotechnology of Sfax, 3038 Sfax, Tunisia*
[2] *Department of Pathology, Habib Bourguiba Hospital, 3029 Sfax, Tunisia*
[3] *Osancor Biotech Inc, Watford, Herts WD17 3BY, UK*

Correspondence should be addressed to Ahmed Rebaï, ahmed.rebai@cbs.rnrt.tn

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We evaluated the association of epidermal growth factor receptor (EGFR) 142285G>A (R521K) and estrogen receptor alpha (ESR1) 2014G>A (T594T) single nucleotide polymorphisms with breast cancer risk and prognosis in Tunisian patients. EGFR 142285G>A and ESR1 2014G>A were genotyped in a sample of 148 Tunisian breast cancer patients and 303 controls using PCR-RFLP method. Immunohistochemitsry was used to evaluate the expression levels of EGFR, HER2, ESR1, progesterone receptor and BCL2 in tumors. We found no evidence for an association between EGFR R521K polymorphism and breast cancer risk. However, we found that the homozygous GG (Arg) genotype was more prevalent in patients with lymph node metastasis ($P = .03$) and high grade tumors ($P = .011$). The ESR1 2014G allele showed significant association with breast cancer risk ($P = .025$). The GG genotype was associated with HER2 overexpression and this association withstood univariate and multivariate analyses ($P = .009$; $P = .021$, resp.). These data suggest that the R521K might be a prognostic factor, because it correlates with both tumor grade and nodule status. The higher expression of HER2 in ESR1 T594T GG patients suggests the possibility that ESR1 gene polymorphisms accompanied by HER2 expression might influence the pathogenesis of breast cancers.

1. Introduction

The epidermal growth factor receptor (EGFR, MIM: 131550), also known as HER-1 or ErbB-1, is the prototype member of the type I receptor tyrosine kinase (TK) family. It is a transmembrane protein with intrinsic tyrosine kinase activity, whose activation leads to downstream effects in gene expression, cellular proliferation, inhibition of apoptosis, and angiogenesis [1]. Exons 1–14 code for the extracellular domain, exon 15 encodes the transmembrane region, and exons 16–20 code for the intracellular tail. EGFR belongs to the ErbB family of TK receptors, whose other members include ErbB2 (HER2/*neu,* MIM: 164870), ErbB3 (HER3, MIM: 190151), and ErbB4 (HER4, MIM: 600543) [2].

A polymorphic variant in the EGFR gene arising from a single nucleotide substitution (142285G>A) leading to an Arginine (R)/Lysine (K) substitution in codon 521 in the extracellular subdomain IV of the EGFR gene (rs11543848, also known as HER-1 R497K) has been identified [3]. Compared with the "wild-type" HER-1 142285G (521R) allele, that the 142285A (521K) variant has attenuated function in ligand binding, growth stimulation, tyrosine kinase activation, and induction of the proto-oncogenes *myc*, *fos*, and *jun* [4].

Among the steroid receptors, estrogen receptor alpha (ERα or ESR1, MIM: 133430) and the ER-regulated progesterone receptor (PGR, MIM: 607311) are of special interest because of their elevated protein levels in some premalignant and malignant breast cells [5]. ERs belong to a family of transcription factors, the nuclear receptor superfamily, responsible for mediating the effects of several hormone families including sex steroids on development, reproduction, proliferation, cellular homeostasis and gene expression [6]. There are two isoforms of ER, ESR1 and

Table 1: Position of SNP and enzyme and primers used in this study.

Genes*	Variation	rs code**	Enzyme	Primer sequences (5′-3′)
EGFR	A/G	rs11543848	MvaI	F 5′-TGCTGTGACCCACTCTGTCT-3′
		(rs2227983)		R 5′-CCAGAAGGTTGCACTTGTCC- 3′
ESR1	A/G	rs2228480	BtgI	F 5′-GAGGAGACGGACCAAAGCCAC-3′
				R 5′-GCCATTGGTGTTGGATGCATGC-3′

*gene names according to gene database in NCBI: http://www.ncbi.nlm.nih.gov/gene.
**rs code in dbSNP http://www.dbsnp.org/.

ESR2 (MIM: 601663), showing 47% sequence identity in the human genome. The ESR1 gene (ERα) located on chromosome 6q25–27 has eight exons and spans more than 140 kilobases. The ESR2 gene (ERβ) located on chromosome 14q23-24 has also eight exons spanning approximately 40 kilobases.

The overexpression of ERs in breast tumour tissues has been demonstrated to be a significant prognostic factor that correlates with higher survival rates and lower risk of relapse [7]. Inherited variants of the estrogen receptor gene have been observed to occur at increased rates in breast cancer [8]. One of the most studied polymorphism is the coding synonymous variant at codon 594 (rs2228480) within the last exon of the gene. This variant is thought to play a role in distinguishing between the receptor agonist or antagonists binding to the receptor molecule [9] and has been suggested, although inconsistently, to be associated with risk of breast cancer [10] and more recently of thyroid cancer [11].

In the present study, we analyzed the two above mentioned polymorphisms (EGFR 142285G>A; ESR1 2014G>A) in 148 breast cancer patients and 303 healthy individuals and investigated their association with breast cancer in order to evaluate their potential as prognostic/predictive factors.

2. Materials and Methods

2.1. Population Samples. The studied population comprised 148 Tunisian women with pathologically proven breast cancer selected from the register of Sfax University Hospital Habib Bourguiba between the years 2001 and 2006. Only patients that have complete clinical records and frozen tumor samples were included in the study. The average age was 49.9 ± 11.4 (range 17–78 years). One hundred twenty nine patients had invasive ductal carcinoma, 6 patients had invasive lobular carcinoma and 10 patients had medullar or tubular cancers. Histological grade was not available for lobular carcinomas since tubule formation is not a characteristic of the tumour.

The protein expression levels of EGFR, HER2, ESR1, PGR, and BCL2 (MIM: 151430) was assessed using immunohistochemistry on frozen tissue. Their primary antibody, (respectively: DM267/ACRIS, BM5084/ACRIS, clone 1D5/DAKO, clone PgR636/DAKO, clone 124/DAKO) were incubated during 30 minutes at room temperature, A biotin-labeled secondary antibody (goat antirabbit, DAKO, 1 : 500) was used for 15 minutes at room temperature. Then the avidin-biotin-HRP complex as directed by DAKO was applicated for 15 minutes, finally, the immunoprecipitate was visualised by treating with diaminobenzidine tetrahydrochloride (DAB) (DAKO) for 30 minutes. The sections were counterstaining with haematoxylin. Immunostaining was considered positive if more than 5% of tumour cells were stained and scored on the basis of the approximate percentage of positive tumours cells and the relative immunostaining intensity [12].

Other variables such as tumour size (in cm), Scarff-Bloom-Richardson (SBR) grade and nodal status were also evaluated. Tumour grades were as follows: well-differentiated collectively referred to as SBR grade I ($n = 9$), grade II ($n = 76$), and grade III ($n = 44$) disease. The nodal status was known for 144 patients; 52 (36.1%) had no nodal involvement, lymph node invasion was detectable in 92 cases (63.8%). 51 had one to three nodes involved and 41 had more than three nodes involved.

The control group comprised 303 individuals with no personal or familial history of cancer. The average age of controls was 61.7 ± 9.7 (range 17–87) years.

2.2. DNA Extraction and Genotyping. DNA was extracted from frozen breast cancer tumours and peripheral blood samples of patients and controls using DNA purification kit (Promega, USA). DNA was also extracted from blood of sixty patients, for whom blood samples were available.

EGFR 142285G>A was genotyped by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method [3] (Table 1). The PCR product (155 bp) was digested by *MvaI* restriction enzyme (Fermentas, LIFE SCIENCES) at 37°C overnight. This restriction enzyme recognizes the sequence CC/WGG. The G-allele carrying PCR product is cleaved twice by the enzyme giving rise to three fragments (38, 50 and 67 bp), whereas the A allele is cleaved only once (38 and 117 bp). Digestion products were separated by electrophoresis on 4% Nusieve ethidium bromide-stained agarose gels and visualized under UV light.

ESR1 2014G>A was analysed using PCR-RFLP [10] (Table 1). The PCR products of 227 (bp) were then digested with BtgI (C/CRYGG) (New England Biolabs, Inc., USA) overnight at 37°C. The sizes of the restriction fragments of PCR product were 227 bp for the wild type homozygote (AA), 129 and 98 bp for the GG genotype and 227, 129 and 98 bp for the heterozygote (AG).

Genotypes of tumour and blood samples from sixty patients were found to be identical. In data analysis, we considered only genotypes from tumours assuming them to be identical to blood DNA genotypes for all patients.

Table 2: Allelic and genotype distribution frequencies of R521K and T594T polymorphism in the patients and the control groups.

Polymorphism	Genotypes			Alleles (%)	
	AA	AG	GG	A	G
EGFR R521K					
Cases ($n = 148$)	12	46	90	70 (23.6)	226 (76.3)
Controls ($n = 303$)	26	103	174	155 (25.6)	451 (74.4)
	AA versus GG	AA versus AG	AA versus (AG + GG)	A versus G	
Odds ratio	1.12	0.97	1.06	1.110	
(95%CI)	[0.54–2.17]	[0.45–2.08]	[0.52–2.17]	[0.80–1.53]	
Chi-square (*P-value*)	.09 (.76)	.01 (.93)	.03 (.86)	.40 (.52)	
ESR1 T594T					
Cases ($n = 142$)	5	36	101	46 (16.3)	236 (83.7)
Controls ($n = 240$)	17	76	147	110 (22.9)	370 (77.1)
	AA versus GG	AA versus AG	(AG + AA) versus GG	A versus G	
Odds ratio	2.33	1.61	1.56	1.54	
(95%CI)	[0.83–6.53]	[0.55–4.71]	[1–2.44]	[1.05–2.25]	
Chi-square (*P-value*)	2.75 (.10)	.77 (.38)	3.812 (.05)	4.96 (.025)	

2.3. Statistical Analysis. Differences in allele and genotype frequencies were evaluated by the Chi-square or Fisher exact test. We used the programs Hwe, Conting and Relrisk from linkage utility programs to test for Hardy-Weinberg equilibrium in control and to calculate χ^2 tests for genotypic and allelic association and odds ratios (OR) with their 95% confidence intervals (http://linkage.rockefeller.edu/).

Association between each of the SNP and clinicopathological parameters was assessed using chi-square test. To study correlation between clinical parameters and genotypes of EGFR and ESR1 polymorphisms in multivariate mode we used binary logistic regression with each parameter as dependent variable and genotypes of the two studied SNP together with other clinical parameters as independent variables. Statistical significance for all tests was declared when P-values are <.05.

3. Results

3.1. Association of Polymorphisms with Cancer Risk. Allelic and genotype frequencies of the two studied polymorphisms in patients (DNA from blood samples) and controls are given in Table 2. Genotype frequencies for rs11543848 and rs2228480 in controls agreed with frequencies expected under the Hardy-Weinberg equilibrium.

Regarding EGFR *R521K*, genotype and allele frequencies were very similar between patients and controls. In both groups, about 60% of individuals were homozygous R/R, 32% were R/K heterozygotes and the remaining 8% carried the K/K genotype.

Concerning the ESR1 codon 594 SNP, we found the frequency of the G allele to be significantly higher in patients than in controls ($P = .025$). The same trend, albeit barely significant, was observed for the GG genotype ($P = .05$) (Table 2). The ESR1 594 SNP provides 71% empirical power for association at 5% significance level calculated as described in Maalej et al. [13].

3.2. Association between SNP and Clinical Parameters in Patients. Table 3 shows a significant association between EGFR R521K genotypes and SBR grade ($P = .011$) and Lymph-node status ($P = .03$). Genotypes RK and KK were more frequent in patients with positive nodal status and KK homozygotes were more prevalent in SBR grade III than in other tumours. In logistic regression analysis, the association between SBR grade and EGFR R521K genotype was even higher after adjustment for other clinical parameters and for the ESR1 2014G>A polymorphism ($P = .008$). The RR genotype seems to be associated with a good prognosis.

No association was found between ESR1 T594T and any of the clinical parameters in this study, except the strong association revealed with HER2 status both in univariate ($P = .009$) and in multivariate analysis using logistic regression with HER2 status as dependent variable adjusting for other clinical variables ($P = .021$). However, multivariate analysis of nodal status detected a significant association with ESR1 T594T genotype ($P = .041$). It should be noted that genotype GG was more prevalent in tumours with positive HER2 compared to those with negative HER2 (82.4% versus 60%, resp.).

4. Discussion

Cancer is a complex disease where genetic mutations, deletions, rearrangement, or deletions as well as gene polymorphisms of other genes may affect not only cancer development but also cancer progression and as a result could influence cancer phenotypes [14]. In this study we investigated two polymorphisms within the EGFR and ESR1 genes that are known to play a key role in breast cancer onset and progression. EGFR is frequently overexpressed in a wide variety of solid tumors and is a target for cancer drugs [15]. The possibility that different genetic polymorphisms in the EGFR gene may regulate, at least in part, EGFR expression and/or activity, is an attractive hypothesis that may help

Table 3: Association between EGFR and ESR1 genotype with EGFR, HER2, ER, PR, and BCL2 protein expression and clinical parameters.

Parameter	Genotype counts EGFR R521K				Genotype counts ESR1 T594T			
	AA	AG	GG	Chi-square *P-value*	AA	AG	GG	Chi-square *P-value*
Tumour size								
≤2 cm	2	5	17	1.172	0	6	17	1.077
>2 cm	9	37	70	*.557*	5	29	78	*.584*
Lymph-node								
Negative	6	9	37	7.022	3	16	31	3.629
Positive	6	35	51	.030	2	19	68	*.163*
Tumour grade								
SBR I/II	9	31	45	9.103	3	18	60	3.080
SBR III	1	8	35	.011	0	14	28	*.214*
Estrogen receptor 1								
Negative –	5	12	26	1.035	2	8	32	1.640
Positive +	6	29	54	.596	2	24	59	*.440*
Progesterone receptor								
Negative –	4	12	39	3.575	2	12	41	.717
Positive +	7	30	46	*.167*	3	22	53	*.699*
BCL2-protein								
Negative –	1	7	18	1.502	1	7	18	.6
Positive +	7	16	35	*.472*	1	13	44	*.741*
HER2-protein								
Negative –	3	13	24	.581	0	16	24	9.428
Positive +	6	15	36	*.748*	2	8	47	*.009*
EGFR-protein								
Negative –	7	12	32	3.112	2	13	36	1.631
Positive +	1	11	21	*.211*	0	7	26	*.442*

to identify patients whose tumours are likely to respond to EGFR-targeted therapies or radiotherapy [16].

Among the most interesting polymorphisms, the EGFR variant R521K has been previously described to be associated with cancer severity in other EGFR expressing tumours, such as gliomas and lung cancer [17]. Patients with rectal cancer carrying the R allele tended to have a higher risk of local recurrence [3, 18].

In our population-based case-control study, we found no evidence for an association between R521K and breast cancer risk. The frequency of the K allele in controls and patients was very similar (25.5% versus 23.6%). According to the SNP database, the frequency of the K allele in our population is similar to that in Europeans (25%). In comparison, its reported frequency in African-American, Subsaharan-African, and Asians is 10.9%, 16% and 45.1%, respectively.

We found a significant correlation between R521K genotype distribution and SBR grades I and II ($P = .011$) and lymph node metastasis ($P = .03$). The association between EGFR variant R521K and lymph node metastasis deserves further consideration in future studies as a clinical indicator during presurgical evaluation; in fact, various studies of lymph node metastasis have considered factors such as intrinsic genetic factors involving cell mobility, vascular invasion and angiogenesis [19, 20].

The R521K EGFR genotype correlates with a decrease in EGFR phosphorylation, decreased invasion, lower nodal involvement, reduced subsequent metastasis, and longer disease-free and overall survival in stage II/III colorectal carcinoma patients who have received curative surgery [18]. Zhang et al. [3] reported that EGFR polymorphism R521K was negatively associated with pelvic recurrence in patients with rectal cancer treated with chemoradiation.

The second SNP that we investigate here is the synonymous codon T594T A/G transition in the ESR1 gene. Estrogen receptor alpha gene (ESR1) has been implicated in the initiation and development of breast cancer. Since it is an important mediator of the hormonal response in estrogen-sensitive tissues, ESR1 polymorphism was postulated to be potential risk factor of breast cancer [21]. We found here a significant association of G variant with breast cancer risk. This result is in agreement with reports in European populations [8] but this association was not reported in the Turkish population [10].

The expression of HER2 was more pronounced in the presence of the ESR1 GG genotype. The cytoplasmic over-expression of HER2 in human breast cancer is considered to be a poor prognostic factor [22]. It was reported that the HER2 gene harbours an estrogen-responsive element ERE [23] in its regulatory region. Since the polymorphism

ESR1 codon 594 was hypothesized to be associated with altered receptor expression and function [24, 25] this might explain its relation to HER2 expression level. The increased expression of ErbB-2 and the EGFR external domain in the presence of the GG genotype might provide an increased risk for breast cancer. Polymorphisms of the ESR1 genes have been suggested to be associated with the occurrence of several disorders [26, 27], including breast cancer [28, 29].

In multivariate analysis a significant association was found between ESR1 codon 594 and lymph node status in Tunisian population. A similar result was reported in Taiwanese population [20]. This suggests that this marker would be a good predictor of nodal status prior to surgical intervention and thereby provides an indicator for deciding whether chemotherapy should be given or not.

5. Conclusion

In summary, in a Tunisian breast cancer patient population, a significant association was found between SNP R521K of EGFR (rs2227983) and SBR grade as well as with Lymph-node status, suggesting that this polymorphism might be clinically important in prognosis. The association of ESR1 polymorphisms with breast cancer risk and HER2 expression but not with other clinical parameters might reflect that it plays a role in the pathogenic processes resulting in the development of breast cancer. With multivariate analysis a correlation between Lymph node metastasis and GG genotype of ESR1 2014G>A (T594T) was found. Validation and functional studies are needed to assess the significance of these associations in clinical practice.

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