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IDENTIFICATION OF G2607A MUTATION IN EGFR GENE WITH A SIGNIFICATIVE RATE IN MOROCCAN PATIENTS WITH NASOPHARYNGEAL CARCINOMA

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Abstract – The epidermal growth factor receptor (EGFR) is involved in the regulation of several cellular processes and in the development of many human cancers. Somatic mutations of EGFR at tyrosine kinase domain have been associated with clinical response to tyrosine kinase inhibitors (TKIs) in lung cancer patients. In this study, we evaluated the frequency of point mutations in EGFR for future use of TKI in clinical treatment of nasopharyngeal carcinoma (NPC). Sixty Moroccan patient specimens of NPC were analysed for EGFR mutations in the region delimiting exons 18 and 21 by direct sequencing. Our results showed the absence of mutations in the EGFR kinase domain in these exons in all 60 analysed specimens. Sequence analysis of the EGFR-TK domain, revealed the presence of (G2607A) polymorphism at exon 20. The genotypes AA and GA were found respectively in 39 (65%) and 16 (26.6%) cases. Statistical analysis showed no difference between the polymorphism and either gender or age of patients. Mutations in EGFR kinase domain are rare events in NPC biopsies, suggesting, that treatment of NPC patients with TKI may not be effective. However, EGFR G2607A polymorphism at exon 20 is frequent in NPC cases and could be associated to clinical response to TKI therapy.

Key words: Nasopharyngeal Carcinoma, EGFR, mutation analysis, polymorphism.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is different from other head and neck cancers because of its specific multifactorial etiology, its geographical distribution and its high sensitivity to radiotherapy and chemotherapy induction. In most parts of the world, this epithelial malignancy has an annual incidence below 1 per 100,000 people. Foci of high incidence (25-80 per 100,000) are located in Southeast Asia, especially in South China, Taiwan, Vietnam, and Philippines (26). In developed countries, Western Europe and United States (US), the incidence of NPC is less than 2 per 100 000 people. There are also large areas of intermediate incidence (3-8 per 100,000) including North African countries (8), Saudi Arabia (14), the Caribbean and the Eskimo population from Alaska and Greenland (11).

Epidemiological and clinical differences between Asian and North African NPCs are reported, suggesting that they don't result from the same combination of etiological factors (5). The main difference relates to their age distribution, which is unimodal in China, with one single incidence peak seen at the age of around 50 years old, but bimodal in the Mediterranean area, with a main peak around 50 years (80% of patients) associated with a secondary peak in the range of 10-25 years (20% of patients) (4, 16). Major risk factors for NPC include Epstein-Barr virus infection, diet and genetic susceptibility (5).

NPC is a radiosensitive disease and radiotherapy remains the mainstay of treatment

for non-disseminated disease (28). The addition of chemotherapy to standard radiotherapy treatment protocols will be of great interest in improving the NPC management and survival rates.

Different studies have supported epidermal growth factor receptor (EGFR) as a potential therapeutic target for NPC treatment. EGFR is overexpressed in 80% of NPC and represents a negative prognostic control (6, 18, 27). Epidermal growth factor receptor, a membrane tyrosine kinase (TK) receptor, play essential role in regulating a number of cellular processes including cell proliferation, survival and migration (9). Dysregulation of its activity has been strongly implicated in the pathogenesis of numerous carcinomas, most notably non small cell lung carcinoma (21). This dysregulation may be a result of mutations in EGFR gene.

EGFR mutations can be classified in three main groups: mutations that lead to changes in the extracellular domain, those that lead to changes in the intracellular domain and those that specifically lead to changes in the intracellular tyrosine kinase (TK) domain. The main interest was focused on the last group where the EGFR mutations are located exclusively in four mutations in the region delimiting exons 18 and 21 (1, 22).

Actually, more than 10 EGFR-targeting agents are in advanced clinical development for the treatment of various human cancer types. However, the interest is focused on two small-molecules, EGFR tyrosine kinases inhibitors (TKIs): gefitinib and erlotinib. These two oral active EGFR TKI have demonstrated long-lasting responses in a sub-population of non–small cell lung cancer (NSCLC) patients who were found to have specific functional EGFR mutations (3, 17, 23). Actually, EGFR mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro* (17, 23, 24). The majority of alterations were found as deletions in exon 19 (56%), followed by point mutations in exon 21 (30%) and the reminder of alterations (14%) were scattered in exons 18 and 20. All of these EGFR mutations affect amino acids near the adenosine triphosphate binding pocket (22). The binding of TKI at this site leads to abrogation of pro-survival signals on which tumour cells carrying the mutant receptor have become dependent (10).

Ma et al., (19) have evaluated the preclinical activity of gefetinib in 4 NPC cell lines. HK1 was the only gefetinib sensitive cell line (IC_{50}=250 nM), the other cell lines (HONE-1, CNE2 and C666-1) were gefetinib resistant with IC_{50} ranging from 15 to 40 µM. Furthermore, none of the 4 cell lines harboured EGFR TK mutations. To our knowledge, the EGFR mutational status in NPC cases is yet unknown. This study was conducted to evaluate the EGFR mutations, using PCR and sequencing, for exploring future use of TKIs in treatment of NPC in Moroccan patients.

MATERIALS AND METHODS

Samples

Sixty NPC samples were obtained from patients coming for treatment at the National Institute of Oncology in Rabat during the year 2008.

DNA extraction was performed on frozen biopsy materiel. The frozen tissue was homogenized and treated for 3 hr with 200 µg/ml proteinease K in 500 µl digestion buffer (50 mM KCl, 10 mM Tris-HCl (pH 8), 0.5% Tween) at 56°C then at 37°C overnight, the enzyme was subsequently inactivated by heating for 10 min at 95°C. The DNA was purified from digested samples using standard phenol-chloroform extraction with ethanol precipitation and resuspended in 50 µl of TE (pH 8). DNA was immediately used for PCR amplification or stored at -20°C until use.

Detection of EGFR mutations

Genetic analysis of the EGFR gene was performed by PCR amplification of exons 18, 19, 20 and 21 that are the more frequent mutated regions in cancers, and direct sequencing of the PCR products (21). Briefly, DNA was amplified in a total volume of 25 µl, containing 1.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 300 nM of each primer, 0.3 units Taq DNA polymerase, and 2 µl of DNA in 1X Taq polymerase buffer. The primers used to amplify exons 18 to 21 of EGFR gene are listed in Table 1. DNA was first denatured at 95°C during 3 min, followed by 35 cycles of PCR with denaturation at 94°C for 30 s, primer annealing for 1 min at 60°C and primer extension for 1 min at 72°C. At the end of the last cycle, the mixture was incubated at 72°C for 10 min. PCR products were purified using the Magnesil yellow solution (Promega) to eliminate the primers used for PCR reactions, then the sequence reaction for each purified product was done in thermocycler. The sequencing reaction was performed in a final volume of 20 µl containing 20 pmol of one primer (forward or reverse), 3 µl of Big Dye (version 1.1) and 2 µl of purified PCR product. Twenty-five cycles were performed: denaturation at 96°C for 10 s, primer annealing at 55°C for 10 s and extension at 60°C for 4 min. To eliminate the excess of labelled ddNTPs, sequencing reaction products were purified using the Magnesil green solution. Direct sequencing of amplified PCR products was performed on an ABI PRISM sequencing apparatus (ABI Prism 310 Genetic Analyser, Applied Biosystem). The sequence alignments were done with the BioEdit Sequence Alignment Editor and analysed using SeqScape software 2.5 (Applied Biosystems).

Statistical analysis

Statistical analyses were performed using Statistica 8 software that uses directly the Yates' chi-square test for small sample size. The associations between the discrete

Table 1. Primers used to amplify EGFR mutations in the region delimiting exons 18 and 21

Exon	Primers	Sequences	PCR product size
18	EGFR18F	5' CAAATGAGCTGGCAAGTGCCGTGT C 3'	400 bp
	EGFR18R	5' GAGTTTCCCAAACACTCAGTGAAAC 3'	
19	EGFR19F	5' GCAATATCAGCCTTAGGTGCGGCTC 3'	372 bp
	EGFR19R	5' CATAGAAAGTGAACATTTAGGATGTG 3'	
20	EGFR20F	5' CCATGAGTACGTATTTTGAAACTC 3'	408 bp
	EGFR20R	5' CATATCCCCATGGCAAACTCTTGC 3'	
21	EGFR21F	5' CTAACGTTCGCCAGCCATAAGTCC 3'	415 bp
	EGFR21R	5' GCTGCGAGCTCACCCAGAATGTCTGG 3'	

variables were assessed using chi-square test. Differences were considered statistically significant for $P \leq 0.05$.

RESULTS

The demographic characteristics of the 60 patients showed that the sex ratio (Male:Female) was 2.15. The mean age of patients was 40 with extreme ages at 10 and 87 years old. The pathological analysis was performed according to the World Health Organization classification and revealed that among the 60 cases, 58 (96.66%) were classified as type 3 (undifferentiated carcinoma) and only 1 case (1.66%) as type 2 (non-keratinizing NPC) and 1 case (1.66%) as type 1 (keratinizing NPC).

Many hot point mutations in the region delimiting exons 18 and 21 in EGFR gene are associated with a long-lasting response to treatment with some active TKIs as gefitinib and erlotinib. In this study, we have focused our interest on identification of mutations in these exons in biopsies from Moroccan patients with NPC. Direct sequencing analysis of exons 18, 19, 20 and 21 didn't show any mutation in all analysed specimens. It's however noteworthy that sequence analyses of the EGFR TK domains in our NPC specimens, revealed the presence of a sequence difference in exon 20 (G2607A). This G-A nucleotide change was previously reported as a single nucleotide polymorphism (SNP, ID: rs 10251977) in the EGFR-TK gene (Figure 1).

The genotypes AA and AG occurred at a significant frequency in NPC cases. Of the 60 patients, 5 (8.33%) had the EGFR polymorphism (GG) whereas 55 (91.6%) cases had the other EGFR genotypes, including 39 (65%) AA and 16 (26.6%) GA.

Distribution of the G2607A polymorphism according to the age of patients and gender is reported in Table 2. Statistical analysis showed that among the 60 patients, the G2607A EGFR polymorphism did correlate neither with gender nor with age.

DISCUSSION

Epidemiologic and experimental evidence accrued over the last two decades in human tumours have confirmed that abnormal EGFR expression or signalling play a critical role in tumour development (1). EGFR is overexpressed in a wide variety of solid tumours, including NPC. Actually, EGFR has emerged as a leading target for the treatment of patients with NSCLC and specific mutations in the EGFR gene, at mutations in the region delimiting exons 18 and 21, may identify lung cancer patients with a good response to the TKIs. In our study, direct sequencing analysis of the 4 exons showed the absence of mutations affecting the EGFR TK domain in all NPC analysed specimens. This result is supported by previous studies reporting that mutations in the EGFR kinase domain are very rare in other human solid tumors. Arias-Pulido et al. (1) have reported the absence of EGFR mutations in exons 19 and 21 in 89 human neoplastic samples of cervical cancer from USA. Furthermore, analysis of 889 tumours, including colorectal (n=391), gastric (n=185), breast (n=73), acute leukemia (n=88), glioblastoma (n=59) and hepatocellular (n=73) tumour samples showed the presence of EGFR mutations in one breast, one colorectal and one glioblastoma cancer sample (2), suggesting that EGFR mutations are rare or occur at a very low frequency in these solid tumours. Conversely, patients with lung cancer show a high frequency (80%) of EGFR TK domain mutations (20). Our results indicate that EGFR mutations are uncommon in NPC patients. Thus, treatment with TKI (gefitinib or erlotinib) seems to be unlikely effective in these patients and targeting the

Table 2. Distribution of the EGFR G2607A polymorphism by sex and age

Factor		GG (%)	AA+GA (%)	*P*
Age	≤ 30	1(1.66)	25(41.66)	0.5297
	> 30	4(6.66)	30(50)	
Sex	Male	4(6.66)	35(58.33)	0.8065
	Female	1(1.66)	20(33.33)	

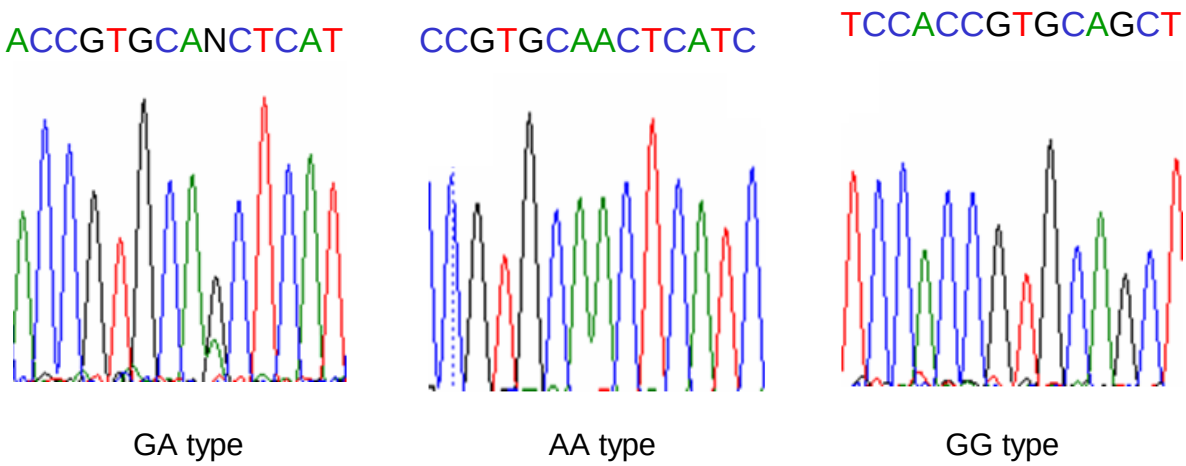

Figure 1. The sequence results of *EGFR* exon 20. Left; heterozygous SNP (GA). Middle; homozygous SNP (AA). Right; wild type (GG).

EGFR with other inhibitors will be more appropriate.

In this study, we detected a polymorphism in exon 20 of the EGFR-TK domain at nucleotide 2607, codon 787 (Gln), which changed nucleotide G to A, without amino acid substitution. The EGFR genotypes GA and AA were significantly higher in NPC patients than the GG polymorphism which is the prototype profile.

The National Cancer for Biotechnology information database reported that there are approximately 563 EGFR SNPs. However, few studies examined the association between EGFR SNPs and human carcinomas (7, 15, 29). Previous reports suggested that EGFR exon 20 mutations have critical role in gefitinib and erlotinib resistance. EGFR containing the exon 20 point mutation T790M were associated with resistance to these two TKI (12, 13).

The EGFR G2607A polymorphism has been previously reported in lung cancer (25) and glioblastoma, with a different frequency distribution (7). To our knowledge, this is the first study to report the presence of this polymorphism in NPC cases.

Zhang et al. suggested that no association was found between the EGFR-TK mutations and the G2607A SNP (29), but Sasaki et al. reported a weak association between G2607A polymorphism and the prognosis of gefitinib therapy in Japanese patients with lung cancer (25). It will be therefore, interesting to evaluate the association between EGFR G2607A polymorphism and clinical response to gefetinib therapy in NPC cases.

Even if the EGFR G2607A polymorphism identified here does not result in amino acid change, this SNP might lead to difference in EGFR gene transcription, mRNA stability or translation, which might interfere with EGFR expression, or could be a genetic marker of another risk-associated genotype.

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