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Original Research

Identification of G2607A mutation in EGFR gene with a significative rate in Moroccan patients with Bladder Cancer

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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 bladder cancer. A total, 50 Moroccan patient specimens with bladder cancer and 48 healthy controls were analysed for EGFR mutations in the region delimiting exons 18-21 by PCR amplification and direct sequencing. Our results showed the absence of mutations in the EGFR kinase domain in these exons in all analysed specimens. However, sequence analysis of the EGFR-TK domain, revealed the presence of (G2607A) polymorphism at exon 20. Statistical analysis showed significant difference in the frequencies of G2607A polymorphism between cancer cases and healthy controls (p=0.0001) and the frequencies of the GG and GA/AA genotypes among the cancer cases were 28% and 72%, respectively. Moreover, allelic frequencies of G2607A polymorphism showed significant difference between cancer cases and healthy controls (p=0.0025). Data analysis showed no significant association between G2607A polymorphism and patients' age, clinical stage and tumor grade ($p > 0.05$). However, a significant difference was found between this polymorphism and patients' sex that could be a sampling bias due to the very limited number of women with bladder cancer. Our findings highlight that, mutations in EGFR kinase domain is a rare event in bladder cancer, suggesting, that treatment of bladder cancer patients with TKI may not be effective. However, the EGFR G2607A polymorphism in exon 20 is frequent in bladder cancer cases and must be further explored for its relevance in the treatment of this disease.

Key words: Bladder Cancer; EGFR; Mutation analysis; Polymorphism; Morocco.

Introduction

Worldwide, bladder cancer is the seventh most common cancer, accounting approximately for 386 300 new cases each year (1, 2). In Morocco, and according to the regional cancer registers, bladder cancer is the most common cancer with an age standardized incidence of 5.8 and 11.3 per 100000 persons in Casablanca and Rabat, respectively (3, 4).

In bladder cancer, a set of clinical and pathological parameters are used for risk stratification, such as number of tumors, size of the tumor, prior recurrence rate, T-stage, presence of *in situ* carcinoma, tumor grade, lymph node status and variant histology. However, there are limited biomarkers for predicting clinical outcomes and for the development of targeted therapies (5).

During last decades, targeted therapy using tyrosine kinase inhibitors (TKI) has emerged as an alternative and effective approach in the treatment of various malignancies with epidermal growth factor receptor (EGFR) mutations. EGFR is a membrane tyrosine kinase receptor that contributes to growth activity and is implicated in the regulation of important processes in carcinogenesis, including cell survival, cell cycle progression, tumor invasion and angiogenesis (6, 7). Currently, different studies have supported EGFR as a potential therapeutic target for bladder cancer treatment and EGFR-TKI treatment was investigated for bladder cancer in several clinical trials (CALBG-90102, NCT00088946, NCT00380029). Actually, more than 10 EGFR-targeting agents are in advanced clinical development for the treatment of various human cancer types and the interest is focused on two small-molecules, EGFR-TKIs: gefitinib and erlotinib. These two oral active EGFR TKI have demonstrated long-lasting responses in a subpopulation of non– small cell lung cancer (NSCLC) patients who were found to have specific functional EGFR mutations in exons 18-21 containing the kinase domain (7, 8). Some TKIs, including gefitinib, dacomitinib and erlotinib, were found to have antitumor activity in preclinical studies in urothelial cancer cell lines (9, 10) and in human bladder cancer models (11).

Bladder cancer is genetically an heterogeneous disease. Recent studies have identified frequent mutations in several targetable kinases including *FGFR3*, *ERRB2* and *PIK3CA* (12, 13). However, mutations in EGFR kinase domain were not found in any bladder cancer cell lines and were reported as a rare event in patient

samples (14-17). Recently, Ross *et al*. have reported two EGFR alterations in bladder cancer cases, including a Q486* mutation in exon 19 (18).

Interestingly, a growing number of studies showed that EGFR genetic polymorphisms could be potential predictive biomarkers of TKIs treatment (19-22) and there is currently increasing interest in SNP mutations in EGFR, given that they could affect the efficacy of EGFR tyrosine kinase inhibitor (TKI) treatment in various cancers (23). Indeed, it is well known that exons not only encode the amino acid sequence of the protein, but also contain sequences that influence translation or mRNA degradation. Thus, EGFR exon SNPs could influence EGFR gene expression and/or protein activity and thereby alter the affinity of EGFR for not just its ligands, but also for anticancer agents that target this protein (24). In this field, two clinical studies showed that SNPs in EGFR exons 18, 19, 21, and 25 affect the clinical efficacy of gefitinib and may be potential biomarkers for the prediction of the clinical outcome of gefitinib-treated patients with advanced non-small cell lung cancer (NSCLC) (21, 25). However, the influence of polymorphisms in EGFR on bladder cancer risk and prognosis has not been investigated extensively. Therefore, we have planned to evaluate genetic variants of EGFR exons 18-21, using PCR and sequencing, and to assess the association between EGFR point mutation and risk of bladder cancer development for the potential use of TKIs in treatment of bladder cancer patients in Morocco.

Materials and Methods

Specimens

Cancer cases. Fifty fresh frozen urinary bladder biopsies were recruited from Urology department of the Military Hospital of Instruction Mohammed V in Rabat, Morocco. Tumor samples were collected by transurethral resection (TUR) or from cystectomy specimens.

The corresponding hematoxylin-eosin-stained sections were examined at the Anatomopathology department at the same hospital; all the samples were confirmed to be histologically urothelial carcinoma (UC) of bladder. Tumors were graded according to the WHO (World Health Organization) criteria (26) and staged according to the TNM (Tumor Node Metastasis) guidelines (27).

Control subjects. A total of 48 healthy peripheral blood samples collected at the National Blood Transfusion Center were used as control. The cancer-free control individuals were frequency matched for age and sex to the cases and were genetically unrelated to the cases.

The study was conducted under the local ethical rules and informed consents were obtained from all patients.

DNA extraction

Genomic DNA was extracted from fresh frozen tissue specimens and from blood samples using the Isolate II Genomic DNA Kit (BIOLINE), according to manufacturer's protocol, and stored at -20°C until use. DNA concentration and purity were evaluated using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific).

Detection of EGFR mutations

Genetic analysis of the *EGFR* gene was performed by PCR amplification and direct sequencing of exons 18, 19, 20 and 21, which are the more frequent mutated regions in cancers (28, 29). Exons DNA were amplified using the primers reported in Table 1. Amplification reactions were performed in total volume of 25 µl. The amplification mixture contained 300 nM of each primer, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 1.5 mM $MgCl_2$, 0.5 unit of Hotstar *Taq* DNA polymerase (Invitrogen, France) and 2 µl of DNA sample in 1x *Taq* polymerase buffer.

DNA was first denatured at 95°C during 7 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. For every reaction, a negative control in which DNA template was omitted from the amplification mixture was included. PCR products were purified using the illustra ExoProStar 1-Step enzymatic clean up system (GE Healthcare Life Sciences) to eliminate the primers used for PCR reactions. Direct sequencing of amplicons was performed using Big Dye Terminator kit (version 3.1) (Applied Biosystem, Foster City, CA, USA) that includes dideoxynucleotides labeled with four fluorochromes of different colors. For each PCR product, both strands were sequenced, in independent reactions, using the mentioned above primers. 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 3.1) 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 labeled ddNTPs, sequencing reaction products were purified by Sephadex G50 chromatography. Direct sequencing of amplified PCR products was performed on an ABI PRISM sequenc-

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

Exon	Primers	Sequences	PCR product size
18	EGFR18F	5' CAAATGAGCTGGCAAGTGCCGTGTC 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'	

ing apparatus (ABI Prism 3130 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 tests were performed using the OpenEpi software. Chi-square test with Yates' correction was used to calculate the p-value. The statistical relationship was considered as significant if the derived p-value was <0.05. The estimated genotypic and allelic frequencies were associated with 95% confidence intervals (CI) calculated using the modified Wald test (Agresti-Coull).

Results

Characteristics of study subjects

The demographic characteristics of the 50 bladder cancer patients showed that 88% were men (n=44) and 12% were women (n=6), the mean age of patients was 49 with extreme ages at 42 and 78 years old. The tumor staging revealed that among the 50 urothelial carcinomas (UC) cases, 31 were classified as pTa-T1 (62%) and 19 (38%) as T2 and beyond (≥T2). The tumor grading showed that 56% of cases were classified as low grade (28/50) and 44% of cases as high grade (22/50).

EGFR mutation analysis of exons 18-21

Successful amplification and direct sequencing of EGFR exons 18-21 was obtained for both bladder cancer specimens and controls. Direct sequencing analysis of exons 18 to 21 demonstrated the absence of mutations affecting the EGFR TK domain in all bladder cases and controls.

Of particular interest, sequence analyses of the EGFR TK domains in our samples revealed the presence of a sequence difference in exon 20 at the position 2607 (G2607A). This silent base substitution (CAG>CAA) in EGFR exon 20 at codon 787 (Q787Q) was previously reported as a single nucleotide polymorphism (rs1050171, NCBI SNP database) in the EGFR-TK gene and the transition of G–A at 2607 nucleotide resulted in three possible genotypes GG, GA, and AA (Figure 1).

Association and stratification analyses between EGFR G2607A polymorphism and bladder cancer risk.

Genotype and allele frequencies of EGFR G2607A polymorphism among bladder cancer cases and control subjects and their associations with bladder cancer risk are presented in Table 2. Statistical analysis showed significant difference in the frequencies of G2607A polymorphism between cancer cases and healthy controls (p=0.0001). Indeed, the frequencies of the GG and GA/AA genotypes among the cases were 28 and 72%, respectively. Conversely, the GG and GA/AA genotypes were 66.67 and 33.33%, respectively, among the controls (P = 0.0001).

On the other hand, analysis of allelic frequencies of G2607A polymorphism showed significant difference between cancer cases and healthy controls (p=0.0025). The 2607A allele was present in 50% of cancer cases and in 20.83% of healthy controls. In contrast, the frequency of 2607G allele was observed in 50% and 79.17% of cancer and healthy control cases, respectively.

Distribution of G2607A polymorphism among the 50 bladder cancer cases and 48 healthy controls according to age and sex is reported in Table 3. Results clearly showed that GA/AA genotypes were significantly associated with an increased risk of bladder cancer in men (p=0031). In women, and despite the low number of recruited cases, it seems that the wild genotype (GG)

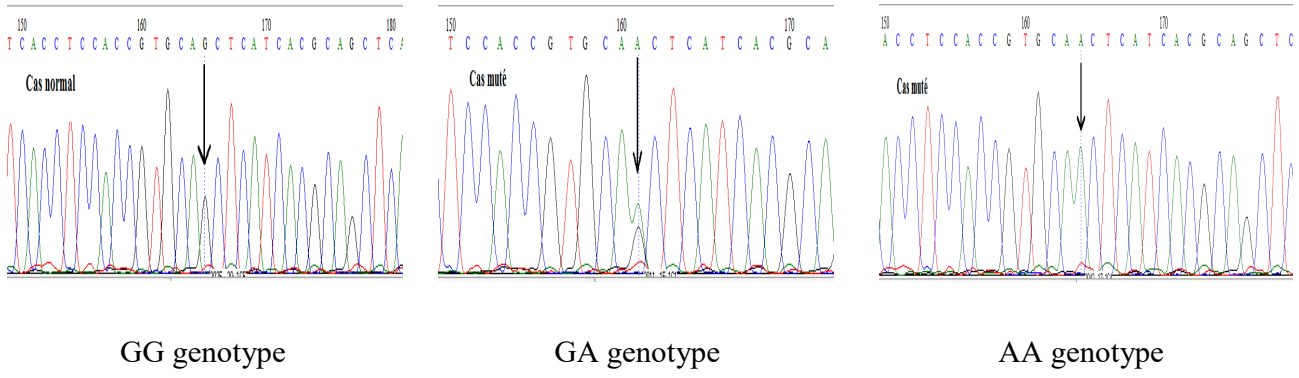

Figure 1. The sequence results of EGFR exon 20. GG genotype: wild type; GA genotype: heterozygous SNP; AA genotype: homozygous SNP.

Table 2. Genotypic and allelic frequencies of G2607A polymorphism in bladder cancer cases and healthy controls.

Cases	N	Genotype		*p*	Allele		*p*
		GG % (95%CI)	GA / AA % (95%CI)		G % (95%CI)	A % (95%CI)	
Bladder cancer	50	28 (17.38 - 41.76)	72 (58.24 - 82.62)	0.0001	50 (36.65 - 63.35)	50 (36.65 - 63.35)	0.0025
Controls	48	66.67 (52.49 - 78.38)	33.33 (21.62 - 47.51)		79.17 (65.55 - 88.46)	20.83 (11.54 - 34.45)	

prevails. A statistically significant association was also observed with the allelic distribution. In fact, the 2607A allele is present in 55.68% of men and only in 8.33 of female (p= 0.0408).

GA/AA genotypes were presents in both young and older bladder cancer cases with no significant difference (p=0.46). G2607A polymorphism was found in 76.92% of bladder cancer cases less than 45 years and 70.27% of bladder cancer cases over 45 years. Moreover, no statistically significant difference was observed between G/A alleles distribution and patients' age (p= 0.5).

In this study, associations between the G2607A polymorphism (Q787Q), and pathological grade and clinical stage of bladder cancer, were also assessed. The distribution of G/A genotypes in the 50 urothelial carcinomas according to clinical stage and tumor grade of bladder carcinoma is reported in Table 4. The SNP, present as heterozygote genotype (GA) or homozygote genotype (AA), prevails in both early (70.97%) and advanced stages (73.68%). Moreover, 2607A and 2607G alleles were equally distributed among early and advanced stages. Statistical analysis showed no significant difference between A/G genotypes and alleles frequencies, and clinical stage of bladder cancer ($p > 0.05$).

In terms of tumor grade, GA and AA genotypes prevail also in both low grade and high grade. In low grades, GG and GA/AA genotypes were detected respectively in 35.71% (10/28) and 64.29% (18/28) of cancer cases. In high grades, GG genotype was detected in 18.18% (4/22) and GA/AA genotype in 81.82% (18/22) of cases. Statistical analysis showed that there's any significant association between G2607A polymorphism and cancer stage (p=0.1469).

Allele frequency analysis showed that the 2607G allele is present in 62.5% of low grade and only in 34.09% of high grade tumors, whereas the GA/AA genotypes were presents in 37.5% of low grade and only in 65.91% of high grade tumors. Statistical analysis showed a weak association between allelic distribution and tumor grade in bladder cancer cases (p=0.0437).

Discussion

Epidemiologic and experimental evidence accrued over the last two decades in human tumors have confirmed that abnormal EGFR expression or signaling play a critical role in tumor development (8). EGFR is overexpressed in a wide variety of solid tumors, including bladder cancer (30). Recently, EGFR has emerged as a leading target for the treatment of patients with NSCLC and specific mutations in the EGFR gene, in the region delimiting exons 18 and 21, may identify lung cancer patients with a good response to the TKIs (31). In this study, we have focused our interest on identification of mutations in the region delimiting EGFR exons 18 and 21 associated with a long-lasting response to treatment with some active TKIs as gefitinib and erlotinib. To our knowledge, this is the first study conducted to evaluate the EGFR status on Moroccan patients with bladder cancer. Direct sequencing analysis of the 4 exons showed the absence of mutations affecting the EGFR TK domain in all bladder cancer specimens. These results are in agreement with previously reported studies showing that mutations in EGFR kinase domain are very rare events in urothelial carcinomas and bladder cancer cell lines (14-18, 32). Furthermore, EGFR mutations are rare or occur at very low frequencies in other solid tumors as cervical cancer (8), colorectal cancer, gastric cancer and glioblastoma (33). Conversely, Asian patients with lung cancer show a high frequency (51%)

Table 3. Genotypic and allelic frequencies of G2607A polymorphism according to age and sex.

UC cases (N= 50)		N	Genotype		p	Allele		p
			GG % (95%CI)	GA / AA % (95%CI)		G % (95%CI)	A % (95%CI)	
Age	≤ 45	13	23.08 (7.499 - 50.94)	76.92 (49.06 - 92.5)	0.4600	53.85 (29.13 - 76.81)	46.15 (23.19 - 70.87)	0.5000
	> 45	37	29.73 (17.37 - 45.9)	70.27 (54.1 - 82.63)		48.65 (33.45 - 64.1)	51.35 (35.9 - 66.55)	
Sex	Male	44	20.45 (10.93 - 34.72)	79.55 (65.28 - 89.07)	0.0031	44.32 (30.68 - 58.86)	55.68 (41.14 - 69.32)	0.0408
	Female	6	83.33 (41.78 - 98.86)	16.67 (1.136 - 58.22)		91.67 (48.5 - 100)	8.33 (0.0 - 51.5)	

Table 4. Genotypic and allelic frequencies of G2607A polymorphism according to clinical stage and tumor grade.

UC cases (N= 50)		N	Genotype		p	Allele		p
			GG % (95%CI)	GA / AA % (95%CI)		G % (95%CI)	A % (95%CI)	
Stage	pTa-T1	31	29.03 (15.94 - 46.75)	70.97 (53.25 - 84.06)	0.4535	51.61 (34.84 - 68.03)	48.39 (31.97 - 65.16)	0.5000
	≥ pT2	19	26.32 (11.45 - 49.14)	73.68 (50.86 - 88.55)		47.37 (27.33 - 68.3)	52.63 (31.7 - 72.67)	
Grade	Low	28	35.71 (20.63 - 54.25)	64.29 (45.75 - 79.37)	0.1469	62.50 (44.05 - 77.93)	37.50 (22.07 - 55.95)	0.0437
	High	22	18.18 (6.709 - 39.11)	81.82 (60.89 - 93.29)		34.09 (17.9 - 55.01)	65.91 (44.99 - 82.1)	

of EGFR TK domain mutations compared with less than 10% of non-Asian patients (34, 35). Our results clearly indicate that EGFR mutations are uncommon in bladder cancer patients, suggesting that treatment in monotherapy with TKI (gefitinib or erlotinib) seems to be unlikely effective in these patients and targeting the EGFR with other inhibitors will be more appropriate.

Nevertheless, a silent base substitution (CAG>CAA) without amino acid substitution in EGFR exon 20 at codon 787 (Q787Q) was found in 72% of cases. This polymorphism is a known single nucleotide polymorphism (SNP), which frequencies vary in different populations, being the G allele more frequent in Asians and African Americans, whereas the A allele is more frequent in Europeans (rs1050171, NCBI SNP database). This polymorphism was also observed, with a higher prevalence, in lung cancer (64.8%) (36), colon cancer (82.7%) (37), hepatocellular carcinoma (81.5%) (38), cervical adenosquamous carcinoma (56%) (39) and glioblastoma (87.8%) (40). Of particular interest, this polymorphism was also reported in pulmonary mucoepidermoid carcinoma, a rare entity of lung cancer, and was found in 60% of cases (41). In Morocco, this polymorphism was reported only in patients with nasopharyngeal carcinoma; the genotype AA being in 65% of cases and GA 26.6% of cases (29).

The EGFR genotypes GA and AA were significantly higher in patients than the prototype profile GG ($p=0.0001$). Nevertheless, no significant association between this polymorphism and patients' age, clinical stage and tumor grade was observed among our cancer cases ($p>0.05$). However, a significant difference was found between G2607A polymorphism and sex. This association must be considered with caution because of the very limited number of female patients, as compared to male patients. Indeed, in Morocco, as it's the case worldwide, bladder cancer appear to have a higher incidence in men than in women, and men are about 3 to 4 times more likely to get bladder cancer during their lifetime than women (3, 4, 42, 43).

The use of EGFR G2607A polymorphism in exon 20 (rs1050171), as biomarker for targeted therapy, was already discussed. This polymorphism was previously reported to be associated with positive response to treatment with tyrosine kinase inhibitors (TKIs) in some cases of small cell carcinoma of the lung (20), and with worse survival rate in some oesophageal squamous cell carcinoma cases (44). More recently, Bonin et al. (2015) have reported that GG genotype of polymorphism rs1050171 seems to be highly predictive of response to anti-EGFR therapy and defines a patients' subpopulation with metastatic colorectal carcinoma having a significantly longer progression free survival (45).

Of particular interest, the efficacy of Gefitinib was reported on both head and neck squamous cell carcinomas and cell lines, exhibiting a higher sensitivity of (G/A) heterozygous when compared with (G/G) homozygous (46, 47).

There is evidence that EGFR G2607A polymorphism is associated with susceptibility to gefitinib, the main TKI used in the cancer therapy, however the mechanism is still unclear. EGFR G2607A polymorphism does not result in amino acid change, but 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. Nakazaki *et al*. have reported that heterozygous mutation (G/G→G/A) at nt 2607 of the EGFR gene is closely associated with increases in EGFR copy number and mRNA half-life, but impaired EGFR protein synthesis in squamous cell carcinomas of the head and neck (47). Other EGFR SNPs, including rs2227983 (R497K) (48-51) and rs2293347 (C2982T) (52), were also discussed and more likely to affect the biological behavior of tumors (such as tumor growth, invasion, metastasis, and progression) than to define susceptibility to cancer development.

In conclusion, the EGFR G2607A polymorphism is frequent in patients with bladder cancer and could be used as a potential key for target therapy with TKIs, and further investigation are needed to explore the relevance of this SNP in the treatment of bladder cancer. In this field, it will be of a great interest to evaluate the association between EGFR G2607A polymorphism and clinical response to gefitinib therapy in bladder cancer cases.

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