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1 TITLE

IMPACT OF SINGLE NUCLEOTIDE POLYMORPHISMS ON THE EFFICACY AND TOXICITY OF EGFR TYROSINE KINASE INHIBITORS IN ADVANCED NON-SMALL CELL LUNG CANCER PATIENTS.

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2 ABSTRACT

EGFR tyrosine kinase inhibitors (EGFR-TKIs) are the treatment of choice for advanced-stage (IIIB-IV) NSCLC patients with mutations in *EGFR.* However*,* EGFR-TKIs clinical outcomes vary from person to person and these inter-individual differences may be due to genetic factors such as single nucleotide polymorphisms (SNPs). SNPs in genes involved in in EGFR-TKIs pharmacodynamics, metabolism and mechanism of action have been demonstrated to be associated with response, survival and toxicity in advanced NSCLC patients treated with EGFR-TKIs.

Here we review the influence of gene polymorphisms in the EGFR pathway on clinical outcome and toxicity to EGFR-TKIs in advanced NSCLC patients. The *EGFR*-216 polymorphism has reported a strong association between response and/or survival to EGFR-TKIs in Caucasian population. Similarly, the effect of EGFR-CA repeats polymorphisms on survival of advanced NSCLC patients treated with EGFR-TKIs have been confirmed both in Caucasian and Asian population. The influence on toxicity of the -216, -191, CA repeats, Arg497Lys and Asp994Asp polymorphisms in *EGFR* have also been confirmed. Polymorphisms in *AKT* (rs1130214 and rs1130233) and *SMAD3* (rs6494633, rs11071938 and rs11632964) have been associated with survival in advanced NSCLC patients treated with EGFR-TKIs. However, data come from a limited number of studies and need to be confirmed.

Finally, polymorphisms in genes coding proteins of the membrane transporters and cytochrome P450 enzymes have been less extensively investigated. There are few studies with small samples, which complicated the generalization of their role in EGFR-TKIs treatment.

3 INTRODUCTION

Lung cancer is one of the most common and lethal types of cancer in both genders, with an approximate incidence of 14% [1]. Based on the latest cancer statistics, around 222.500 new cases (116.990 in male and 105.510 in female) and 155.870 deaths (84.590 in male and 71.280 in female) are expected to occur in the United States in 2017 [1].

There are two main types of lung cancer: small cell (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts with 80-85% of all lung cancer cases and is classified into three different subtypes: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. In accordance with the American Joint Committee on Cancer (AJCC), the majority of the patients are catalogued as advanced stage (IIIB-IV) at the time of diagnosis [2-4].

For many years, platinum-based chemotherapy has been the treatment of choice for advanced-stage (IIIB-IV) NSCLC [5]. Nevertheless, targeted therapy has emerged as a therapeutic option for selected patients. Patients with somatic, activating mutations in *EGFR* (epidermal growth factor receptor) are treated with an EGFR tyrosine kinase inhibitor (EGFR-TKI), such as gefitinib or (Iressa®; AstraZeneca, London, UK), erlotinib (Tarceva®; Hoffmann-La Roche, Basel, Switzerland), afatinib (Giotrif®; Boehringer Ingelheim, Ingelheim, Germany) or osimertinib (Tagrisso ®; AstraZeneca, London, UK) [6-10]. Activation of the EGFR pathway is induced by ligand binding, which results in receptor dimerization and phosphorylation of tyrosine residues located at the cytoplasmic tail of the receptor, leading to phosphorylation of effector proteins [11,12] (Figure 1). Subsequently, downstream cascades, including the anti-apoptotic Ras signal transduction cascade (KRAS-BRAF-MEK-ERK pathway), the phosphatase and phosphatidylinositol 3-kinase / tensin homolog /v-akt murine thymoma viral oncogene (PI3K/PTEN/AKT), phospholipase C gamma protein pathway, and the STAT signaling pathway are activated, leading to cell proliferation, angiogenesis, migration, survival, and adhesion [13] (Figure 1). EGFR-TKIs are orally active compounds that act by binding to the adenosine triphosphate (ATP)-binding domain of EGFR. The inhibition of the receptor leads to a blockade

of downstream cascades, which induce cancer cell death in EGFR mutated cancer cells [14]. There are two type of EGFR-TKIs that differ in their abilities to fit in the ATP-binding pocket of EGFR. First generation or reversible inhibitors, such as gefitinib and erlotinib, compete with ATP molecules that recognize the kinase active conformation, whereas second generation or irreversible inhibitors such as afatinib, bind to the kinase active site covalently by specifically reacting with a nucleophilic cysteine residue [15]. Third generation inhibitors, such as osimertinib, are irreversible EGFR-TKIs selective for both *EGFR* sensitizing mutations and *EGFR* Thr790Met resistance mutation [16].

Activating mutations in the *EGFR* gene appear more frequently in adenocarcinoma subtype, females, non-smokers and Asians [17-20]. The most frequent mutations in EGFR are small in-frame deletions in exon 19 and a point mutation that replaces an arginine with a leucine at codon 858 (L858R) of exon 21 [21]. Several studies have compared first line EGFR-TKIs versus standard chemotherapy in patients with EGFR mutation-positive tumors, showing longer progression-free survival (PFS) (9.7 months vs 5.2 months), higher overall response rate (ORR) (71.2% vs 47.3%), a more favorable toxicity profile (28.7% vs. 61.0%) and better quality of life (48.0% vs. 40.8%) [8,22]. However, numerous studies have reported significant inter-individual differences in clinical outcomes to EGFR-TKIs, which may be due to genetic factors such as single nucleotide polymorphisms (SNPs) in particular genes [23].

At this respect, the influence of some SNPs in the *EGFR* gene itself have been extensively investigated (Table 1). As described above, AKT pathway also plays an important function on cancer cell proliferation and survival has been reported that SNPs in this gene may dysregulate signaling, promote tumorigenesis and contribute to individual variation in the response and toxicity to EGFR-TKIs [24,25].Finally, other pathways and proteins are also involved in toxicity and response to EGFR-TKIs including, the transforming growth factor beta (TGF-β) pathway, drug transporters, and the cytochrome P450 family. Acting in an opposite way, the TGF-β signaling pathway exerts a robust antiproliferative function [26] and polymorphisms in the genes of pathway may have an effect in the development of toxicity and disease progression to EGFR-TKIs. Genetic alterations in ATP-binding cassette, sub-family B (MDR/TAP), member 1 (*ABCB1*, also called *MDR1*) and ATP binding cassette subfamily G member 2 (*ABCG2*) have also been suggested as predictive markers of clinical outcomes and toxicity to EGFR-TKIs [27]. Finally, EGFR-TKIs are metabolized by members of cytochrome P450 family, mainly by CYP3A4/5, CYP2D6 and CYP1A1. Therefore, SNPs in these genes may modulate enzymatic activities and consequently act as pharmacogenetics predictors of response and toxicity to EGFR-TKIs.

4 EGFR PATHWAY

The most investigated polymorphisms in *EGFR* are rs712829 (G→T substitution at -216 upstream from the initiator codon), rs712830 (C→A substitution at -191 upstream from the initiator codon) and rs11568315 (CA simple sequence repeat in intron 1). *EGFR*-216 and -191 polymorphisms have been described to modulate "in vitro" the expression of *EGFR* gene [28]. *EGFR*-216 is located in a SP1-binding site, a transcription factor required for *EGFR* expression, and the substitution of G to T at this position has been shown to increase the promoter activity by 30% and the *EGFR* mRNA expression by 40% [28]. *EGFR*-191, located four nucleotides upstream of one of six transcription initiation sites, also modulates promoter activity, but not to the same extent as the *EGFR*-216 [28]. In addition, the length of the CA repeat has shown an inverse correlation with the expression of *EGFR* mRNA [29].

The relationship between *EGFR* polymorphisms and clinical outcomes have been investigated extensively. The *EGFR*-216 polymorphism has showed better overall survival (OS), PFS and ORR for patients with T allele (Table 1). For EGFR-191 polymorphism no association with clinical outcomes have been found (Table 1). However, a study in 175 Caucasian stage IB-IV NSCLC

patients evaluated *EGFR* -216G/-191C haplotype (G-C; *EGFR**1) and reported that the absence of EGFR*1 was associated with significantly better OS (HR=0.54; 95%CI=0.32, 0.91; non-EGFR*1 vs EGFR*1) and PFS (HR=0.65; 95%CI=0.42, 0.99; non-*EGFR**1 vs *EGFR**1) [30]. Regarding *EGFR* rs11568315 polymorphism, advanced NSCLC patients with shorter intron 1 CA repeats (<16 CA) of the *EGFR* gene showed an improved response, OS and PFS (Table 1).

An association between *EGFR* polymorphisms and toxicity have also been found in several studies. The T allele for *EGFR*-216 has been associated with skin rash and diarrhea (Table 1) [31,32]. In the case of *EGFR*-191, the A allele has been associated with diarrhea in 80 NSCLC patients [32]. In contrast, no association with toxicity was found after evaluating *EGFR* -216G/-191C haplotype in 109 Caucasian stage IIIA-IV NSCLC patients [33]. For *EGFR* rs11568315 polymorphism, a study with 52 Asian stage IIIB-IV reported that those with longer intron 1 CA repeats (>16 CA) of the *EGFR* gene showed a decreased risk to develop skin rash [34].

Two additional polymorphisms in *EGFR,* rs11543848 (G→A nonsynonymous substitution at codon 497, exon 13, Arg→Lys, Arg497Lys) and rs2293347 (G→A synonymous substitution at codon 994, exon 25, Asp→Asp, Asp994Asp), have also been investigated [35]. The A allele for *EGFR* rs11543848 seems to decrease the activity of EGFR [36,37]. *EGFR* rs2293347 does not change amino acid sequence of the protein and, to date, the possible functionality of this genetic alteration has not been evaluated. Nevertheless, synonymous polymorphisms may affect mRNA stability, translational kinetics, and splicing, resulting in alteration of protein amount, structure or function [38]. Both polymorphisms have been correlated with clinical outcomes in NSCLC patients treated with EGFR-TKIs [25,33,39-43].The A allele for *EGFR* Arg497Lys polymorphism has been associated with longer OS in 225 Asian stage I-IV NSCLC patients with positive lymph node metastasis and previous platinum-based chemotherapy (Log-rank test, p = 0.0072 and p= 0.0038, respectively) [39]. A correlation between *EGFR* Arg497Lys-A allele and lower skin toxicity has also been reported in 96 Caucasian stage IIIB-IV NSCLC patients [33]. In contrast, the GG genotype has been associated with higher diarrhea IN [25]. No association between *EGFR* Arg497Lys polymorphism and ORR has been found [25,40]. Regarding *EGFR* Asp994Asp polymorphism, its association with clinical outcome to EGFR-TKIs remains unclear (Table 1), with some studies reporting better ORR in patients carrying the A allele [41] and others in patients with the G allele [42]. The same contradictory results have been reported in the case of PFS and OS, with some studies finding an association of the GG genotype with a better outcome and others reaching opposite conclusions (Table 1) [41-43].

5 AKT PATHWAY

Three SNPs for *AKT* have been studied; namely G→T,rs1130214; A→G, rs1130233 and C→T rs3730350. A Caucasian study with 230 advanced NSCLC patients treated with erlotinib, gefitinib or icotinib reported that patients with AKT rs1130214-GG genotype had longer PFS than those with the GT and TT genotypes (HR=1.39; 95%CI=0.92, 1.95 for TT vs GG)[24]. For AKT rs1130233, the AA genotype was associated with shorter PFS (p=0.04) and OS (p=0.007) in 96 advanced NSCLC patients treated with gefitinib [25]. No association has been found between AKT rs3730350 and clinical outcomes in 96 Caucasian stage IIIB-IV advanced NSCLC patients treated with EGFR-TKIs [25].

6 TGF-B PATHWAY

The TGF-β signaling may function both as a tumor suppressor and as a tumor promoter pathway in a context-dependent manner via acting on SMAD transcriptional regulators [44]. This behavior depends on cell type and clinical stage of the tumor [44].

Three polymorphisms in *SMAD3* (C→T, rs6494633; C→T, rs11071938 and C→T, rs11632964) were found to be associated with survival in 106 Asian stage IIIB-IV EGFR mutated NSCLC patients treated with EGFR-TKIs [45]. The rs649446633-CC, rs11071938-CT and rs11632964-CT

genotypes were associated with better PFS (HR=0.55; $CI_{95\%}$=0.37, 1.00 for CC vs CT/TT; HR=1.75; $CI_{95\%}$=1.06, 2.89 for CC vs CT/TT and HR=3.01; $CI_{95\%}$=1.54, 5.86 for CC vs CT/TT, respectively) [45]. The CT genotype in the *SMAD3* rs11632964 polymorphism was also associated with longer OS (HR=2.38; $CI_{95\%}$=1.15, 4.94 for CC vs CT/TT) [45].

7 CELLULAR EFFLUX TRANSPORTERS

ABCB1 and ABCG2 are considered the main EGFR-TKIs efflux transporters [46,47]. Polymorphisms in these genes have been shown to alter protein expression and/or activity of these transporters [48-56]. Thus, ABCB1 and ABCG2 polymorphisms may modify the elimination of EGFR-TKIs from the body and as a result affect treatment outcome.

7.1 ABCB1

ABCB1 belongs to the ATP-binding cassette family and plays an essential function on efflux and distribution of many drugs, including EGFR-TKIs [57,58]. Polymorphisms in this gene have been associated with lower expression and function of the ABCB1 protein, resulting in increased extracellular levels of drugs [51-54]. Despite of this key role, none of the polymorphisms studied to date in the *ABCB1* gene (C→T, rs1045642; G→T/A, rs2032582; C→T, rs1128503) have shown a significant association with toxicity in NSCLC patients treated with EGFR-TKIs [27,59]. However, significant differences in toxicity have been demonstrated according to ABCB1 haplotype. A study with 50 Asian stage III-IV NSCLC patients treated with erlotinib have reported that the *ABCB1* rs1045642-TT; rs2032582-TT; rs1128503-TT haplotype was associated with higher plasma concentration of EGFR-TKI and the risk of developing higher toxicity [27]. The influence of these haplotypes on ORR, PFS and OS has not been determined.

7.2 ABCG2

ABCG2 is another member of the ATP-binding cassette family [60]. Genetic alterations in this gene have been has been associated with markedly decreased levels of ABCG2 protein expression and/or activity [48-50,55,56], which increases oral bioavailability of EGFR-TKIs [61]. A great variety of polymorphisms in *ABCG2* gene have been studied such as C→T, rs2622604; C→A, rs2231142; G→A, rs2231137, G→A, rs7699188 and C→T, rs72552713. Nevertheless, none of them have shown a significant association with clinical outcomes in NSCLC patients treated with EGFR-TKIs [62,63]. Only the A allele for rs2231137 has been correlated with grade 2 or worse skin rash in 83 Asian stage I-IV NSCLC patients treated with gefitinib (p=0.046) [59].

8 CYTOCHROME P450 FAMILY

EGFR-TKIs are metabolized in the liver by cytochrome P450 enzymes (CYPs), primarily by CYP3A4/5, CYP2D6 and CYP1A1 [64-67]. Polymorphisms in these genes may alter the metabolic activities of these enzymes and thereby drastically influence EGFR-TKIs plasma concentrations and detoxification, resulting in individual variation in response and toxicity to EGFR-TKIs [40,67-69].

8.1 CYP3A4/5

CYP3A4 and CYP3A5 are key enzymes for EGFR-TKIs metabolism [64-67]. To date, 34 CYP3A4 alleles (haplotypes) have been published on the Human Cytochrome P450 Allele Nomenclature Committee homepage [70]. However, their effects on outcome to EGFR-TKIs has not been investigated. Only the *CYP3A4**1/*1G polymorphism (G→A, rs2242480), within intron 10 of the *CYP3A4* gene, has been studied in 31 Asian stage IIIB-IV NSCLC patients treated with gefitinib but no significant differences in toxicity was found [68]. For CYP3A5, 11 haplotypes have been described but only the *CYP3A5**3 (A→G, rs776746) polymorphism, within intron 3 of the *CYP3A4* gene has been studied in 31 Asian stage IIIB-IV NSCLC patients treated with gefitinib [68]. Nevertheless, no significant association between *CYP3A5**3 polymorphism and EGFR-TKIs was

found [68]. The relationship between both SNPs with response and survival has not been evaluated.

8.2 CYP2D6

CYP2D6 also plays a minor role on EGFR-TKIs metabolism [64-67]. A total of 109 CYP2D6 alleles have been described so far, but only the CYP2D6*1, *2, *3, *4, *5, *6, *9, *10 and *41 alleles have been studied. In 30 healthy volunteers treated with gefitinib [70], those with the CYPD6 extensive metabolizer genotype (*1/*4, *1/*2, *2/*4, *1/*3, *2/*5, *2/*41) presented higher gefitinib plasma concentration in comparison with those with CYPD6 poor metabolizer genotype (*4/*4, *4/*5, *3/*4, *4/*6, *3/*5, *4/*4x2) [67]. Two studies in Asian NSCLC patients treated with gefitinib have also evaluated the effect of CYPD26 (*5 and *10) polymorphisms on gefitinib toxicity but no significant differences were found in the frequency of diarrhea, skin rash, or hepatotoxicity among the genotypes of these polymorphisms [68,69]. Currently, no data are available regarding the influence of these SNPs on response and survival to EGFR-TKIs.

8.3 CYP1A1

CYP1A1 is a major enzyme involved in EGFR-TKIs metabolism [64-67]. Based on the CYP450 database, 13 CYP1A1 haplotypes has been described but only CYP1A1*2A (T→C substitution at 3' non-coding region) and CYP1A1*2C (A→G substitution at exon 7, Val→Ile) have been examined in NSCLC patients treated with EGFR-TKIs [40]. Both CYP1A1*2A and CYP1A1*2C alleles have been associated with increased enzyme activity [71,72]. An Asian study with 115 advanced NSCLC patients treated with an EGFR-TKI reported that patients with CYP1A1*2A-TT had an improved response (p=0.011; TT vs CT/CC) and OS (HR=0.48; $CI_{95\%}$=0.31, 0.73 for TT vs CT/CC) to EGFR-TKI [40]. However, for CYP1A1*2C, no association with clinical outcome for patients treated with EGFR-TKIs has been reported [40]. Finally, no studies have evaluated polymorphisms in *CYP1A1* and their associations with toxicity.

9 CONCLUSIONS

The influence of gene polymorphisms in the EGFR pathway on clinical outcome and toxicity has been extensively investigated in advanced NSCLC patients treated with EGFR-TKIs. The *EGFR*-216 polymorphism have reported a strong association between response and/or survival to EGFR-TKIs in Caucasian population. Similarly, the positive effect of EGFR-CA repeats polymorphisms on survival of advanced NSCLC patients treated with EGFR-TKIs have been confirmed in both Caucasian and Asian population. The influence on toxicity of the -216, -191, CA repeats, Arg497Lys and Asp994Asp polymorphisms in *EGFR* have also been confirmed both in Caucasian and Asian population.

Polymorphisms in *AKT* (rs1130214 and rs1130233) and *SMAD3* (rs6494633, rs11071938 and rs11632964) have been associated with survival in advanced NSCLC patients treated with EGFR-TKIs. However, data come from a limited number of studies and need to be confirmed.

Finally, polymorphisms in genes coding proteins of the membrane transporters and cytochrome P450 enzymes have been less extensively investigated. There are few studies with small samples, which complicated the generalization of their role in EGFR-TKIs treatment.

In summary, polymorphisms in genes most extensively studied such as *EGFR* are promising biomarkers for the selection of treatment and follow-up of NSCLC patients. In clinical practice, *EGFR* polymorphisms may serve as a useful source of information to predict those patients with better response, higher survival and lower toxicity. Therefore, these biomarkers could be a valuable tool for patient stratification. However, polymorphisms in *AKT, SMAD3, ABCB1, CYP3A4, CYP3A5, CYP2D6, CYP1A1* genes need further examination in larger samples (stratified by gender, age and smoking status) and longer follow up.

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