INDEX

1 TITLE

PHARMACOGENETICS OF PLATINUM BASED CHEMOTHERAPY: IMPACT OF DNA REPAIR AND FOLATE METABOLISM GENE POLYMORPHISMS ON PROGNOSIS OF NON-SMALL CELL LUNG CANCER PATIENTS

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CONFLICT OF INTEREST

The authors declare that there is not conflict of interest that could be perceived as prejudicing the impartiality of the research reported and there is not any competing financial interest in relation to the work described in this article.

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2 ABSTRACT

Chemotherapy based on platinum compounds is the standard treatment for NSCLC patients with EGFR wild-type, and is also used as second line in mutated EGFR patients. Nevertheless, this therapy presents poor clinical outcomes. *ERCC1, ERCC2, XRCC1, MDM2, MTHFR, MTR* and *SLC19A1* gene polymorphisms may contribute to individual variation in response and survival to platinum-based chemotherapy. The aim of this study was to investigate the influence of these polymorphisms on response and survival of NSCLC patients treated with platinum-based chemotherapy. A retrospective-prospective cohorts study was conducted, including 141 NSCLC patients. Polymorphisms were analyzed by PCR Real-Time with Taqman® probes. Patients with *ERCC1* rs3212986-GG (p=0.0268; OR=2.50; $CI_{95\%}$=1.12-5.69) and *XRCC1* rs25487-GG (p=0.0161; OR=2.99; $CI_{95\%}$=1.26-7.62) genotype showed significantly better ORR. Cox survival analysis revealed that patients carrying the *MDM2* rs1690924-GG genotype (p=0.0345; HR=1.99; $CI_{95\%}$=1.05-3.80) presented higher risk of death. Furthermore, carriers of *MTR* rs1805087-A alleles (p=0.0060; HR=8.91; $CI_{95\%}$=1.87-42.42) and *SLC19A1* rs1051266-AA genotype (p=0.0130; HR=1.74; $CI_{95\%}$=1.12-2.68) showed greater risk of progression. No influence of *ERCC1* rs11615, *ERCC2* rs13181, *ERCC2* rs1799793, *XRCC1* rs1799782, *MDM2* rs1470383, *MTHFR* rs1801131 and *MTHFR* rs1801133 on platinum-based chemotherapy clinical outcomes was found. In conclusion, our results suggest that *ERCC1* rs3212986, *XRCC1* rs25487, *MDM2* rs1690924, *MTR* rs1805087 and *SLC19A1* rs1051266 gene polymorphisms may significantly act as predictive factors in NSCLC patients treated with platinum-based chemotherapy.

3 INTRODUCTION

Lung cancer is the highest mortal cancer among both genders, being responsible for 27% of all cancer deaths [1]. This type of tumor is the second most diagnosed (after prostate in men and breast cancer in woman), showing 14% of incidence [1]. In accordance with the latest cancer statistics, around 224,300 new cases and 158,000 deaths are expected to occur in the United States in 2016 [1].

Non-small cell lung cancer (NSCLC) is the lung cancer subtype diagnosed in up to 85% of all cases. NSCLC and is classified in three 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].

Chemotherapy based on platinum compounds is the standard treatment for NSCLC patients with EGFR wild-type, and is also used as second line in mutated EGFR patients [5]. This therapy is usually given combined with third-generation drug such as, anti-microtubule agents (taxanes and vinca alkaloids), antifolate agents (pemetrexed), or pyrimidine antagonists (gemcitabine). Chemotherapy versus best supportive care has showed improvement in terms of survival (10.7 months vs 3.9 months, respectively; p<0.001) and symptom control compared with best supportive care [6, 7]. Nevertheless, the overall response rate (ORR) to platinum-based regimen is about 13-47.2% and only 16% of the patients are alive five years after diagnosis [8-24]. The knowledge of predictive and/or prognostic factors may improve clinical outcomes of NSCLC therapy, by stratifying patients into subgroups that could be managed differently. The crucial factor for NSCLC prognosis is the cancer stage, but a significantly variability in terms of response and survival has been described among patients with the same stage of disease, suggesting that other factors may play a role on NSCLC prognosis [4, 25]. Interestingly, genetic factors such as single nucleotide polymorphisms (SNPs) have demonstrated to be associated with inter-individual differences in response and survival in NSCLC patients [26-42].

The main platinum drugs compounds used in NSCLC therapy are cisplatin and carboplatin. They share the same mechanism of action, interfering with DNA and forming DNA adducts,

which induce severe local distortions of the DNA double helix [43, 44]. In response to this DNA damage, various signaling pathways are activated, such as DNA repair and p53 pathways [45-47]. The DNA repair pathways are responsible for detecting and repairing these damaged DNA. Nucleotide-excision repair (NER) and base excision repair (BER) are the two most important DNA repair pathways and are comprised by several proteins, such as excision repair cross-complementing group 1 (ERCC1), excision repair cross-complementation group 2 (ERCC2, also known as XPD) and X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) [45, 46]. The p53 pathway is involved in modulating cell cycle and apoptosis [47]. The principal antagonist of TP53 tumor suppressor gene is *MDM2* proto-oncogene, E3 ubiquitin protein ligase (MDM2), that induces its ubiquitination and degradation [48]. Genetic variants in these genes have showed to be associated with variability in clinical outcomes for NSCLC patients treated with platinum-based chemotherapy [26-33].

Other pathways have also demonstrated to be connected with effectiveness of platinum compounds, such as folate metabolism [34-41]. Folate metabolism is involved in DNA methylation through methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MTR) enzymes. Genetic alterations in these genes disturb methylation of DNA and tumor-suppressor genes, which may influence the clinical outcomes of platinum-based chemotherapy [34-36, 39]. Other gene with a crucial function on folate metabolism is solute carrier family 19 (folate transporter), members 1 *(SLC19A1)*. This transporter is involved in the intracellular uptake of pemetrexed, a drug that is usually given in combination with platinum compounds [39, 49]. Genetic variants in this gene may modify pemetrexed transport and consequently affect the effectiveness of pemetrexed-based chemotherapy [37-41].

In this study, we investigated the association between clinical outcomes of platinum-based chemotherapy and genetic alterations in *ERCC1, ERCC2, XRCC1, MDM2, MTHFR, MTR and SLC19A1* genes in NSCLC patients. To determine the impact of the tumor biology, we also performed a subgroup analysis according to *EGFR* status.

4 MATERIAL AND METHODS

A retrospective-prospective cohorts study was conducted.

4.1 Ethics statement

This study was approved by the Complejo Hospitalario Universitario de Granada (CHUG) Ethics and Research Committee and was performed in accordance with the declaration of Helsinki. Patients signed a written informed consent form for blood or saliva sample collection and genotyping analysis. Sample identification was based on non-patient codes.

4.2 Study population

One hundred and forty-one patients diagnosed with NSCLC between 2003-2015, at the CHUG, Granada, Spain were enrolled in the study and followed up until February 2016. Patients who were age ≥18 years, histologically or cytologically diagnosed NSCLC (stages I-IV), confirmed adequate bone marrow reserve (hemoglobin ≥9g/dl, neutrophil count ≥1500 cells/mm^3, and platelet count ≥100.000 cells/mm^3), adequate liver (bilirubin ≤1.5 mg/dl, aspartate aminotransferase and alanine aminotransferase ≤2.5 times the upper limit of normal) and renal function (creatinine level ≤1.5 mg/dL) and measurable disease by computed tomography of the thorax and abdomen were suitable for the study.

The 141 NSCLC patients were treated with cisplatin or carboplatin intravenously in combination with a third-generation drug (gemcitabine, paclitaxel, pemetrexed and vinorelbine) according to the National Comprehensive Cancer Network version 4.2016 guidelines [5]. Hematology and biochemistry analyses were done at the end of each cycle.

The status of epidermal growth factor *(EGFR)* was analyzed by cobas® EGFR Mutation Test [50].

4.3 Sociodemographic and clinical variables

Sociodemographic data including gender, family history of cancer, previous non-lung cancer, previous lung disease, smoking status and age at diagnosis was collected from clinical records. Clinical data were also collected from clinical records and comprised tumor histology and stage, chemotherapy agents, surgery, radiotherapy and EGFR status. The tumor staging was performed based on AJCC cancer staging manual [51].

4.4 Genetic variables

4.4.1 DNA isolation

Blood samples (3 ml) were collected in BD Vacutainer® K3E Plus Blood Collection Tubes. Saliva samples were collected in 50 ml BD Falcon™ conical tubes (BD, Plymouth, UK). DNA isolation was performed using the QIAamp DNA Mini Kit (QiagenGmBH, Hilden, Germany) according to the manufacturer's instructions for DNA purification from blood or saliva and stored at -40ºC.

4.4.2 Detection of gene polymorphisms

ERCC1 C118T (rs11615), *ERCC1* C8092A (rs3212986), *ERCC2* Lys751Gln (rs13181), *ERCC2* Asp312Asn (rs1799793), *XRCC1* Arg194Trp (rs1799782), *XRCC1* Gln399Arg (rs25487), *MDM2* (rs1470383), *MDM2* (rs1690924), *MTHFR* A1298C (rs1801131), *MTHFR* C677T (rs1801133), *MTR* (rs1805087) and *SLC19A1* Arg27His (rs1051266) gene polymorphisms were analyzed by Real-Time PCR using TaqMan® probes. Genotyping methodology was previously described [52].

4.4.3 Response variables

Platinum-based chemotherapy response was evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (Version 1.1) [53].

Patients classified as Complete Response (CR) + Partial Response (PR) were catalogued as responders to treatment and Stable Disease (SD) + Progressive disease (PD) as non-responders.

4.4.4 Survival variables

Survival was evaluated through OS and PFS, which were measured as follows:

OS as time from cancer diagnosis until final follow-up or death

PFS as the time from initiation of treatment to relapse, death or last known follow-up.

Mortality related data were collected from clinical records and the population-based Cancer Registry of Granada.

4.5 Statistical Analysis

Deviation from Hardy Weinberg equilibrium and pairwise linkage disequilibrium for each polymorphism were calculated using the free, open-source whole genome association analysis toolset PLINK [54].

Quantitative data were estimated as the mean (± standard deviation) for normally-distributed variables or medians and percentiles (25 and 75) for non-normal distributed variables. The Shapiro-Wilks test was used to assess normality.

The bivariate association between response with demographic, clinical and genetic variables was tested using the Pearson's chi-square or Fisher's exact test, and evaluated by relative risk (RR) and their corresponding 95% confidence intervals (CI).

The Kaplan-Meier method and the log-rank test were employed to assess associations between survival with demographic, clinical and genetic variables.

All clinical and genetic variables associated with outcomes in bivariate analysis were tested as potential confounding factors in multivariate analysis. Multivariate Cox proportional hazard regression model (backward stepwise method) was used to estimate the adjusted hazards ratio (HR) and 95% confidence interval ($CI_{95\%}$) for potential predictive factors for survival. The influence of SNPs and clinical variables on response was analyzed using a multivariate logistic regression model (backward stepwise method).

All tests were two-sided and a probability of 0.05 or smaller was considered statistically significant. Data analysis was performed using R 3.0.1 [55].

5 RESULTS

5.1 Patients characteristic

A total of 141 patients with cytologically or histologically confirmed NSCLC were recruited in the study. The baseline characteristics are listed in Table 1. The median age was 61 [52, 67] years, and 104 were males (104/141; 73.76%); 70.5% presented advanced stage (IIIB-IV) (98/139). All patients were treated with platinum-based chemotherapy in addition to one third-generation chemotherapy drug, such as gemcitabine (21/141; 14.89%), paclitaxel (33/141; 23.40%), pemetrexed (37/141; 26.24%) or vinorelbine (50/141; 35.46%).

Response to treatment (CR + PR) was shown in 98 patients (98/141; 70.5%). Regarding treatment options, response to chemotherapy was higher in those patients who received adjuvant chemotherapy (93.8%; 15/16), whereas as response to chemotherapy given as palliative treatment was 50.8% (31/61) (Table 1). During follow-up, 75 death events were documented. Median OS and PFS were 32.2 [27.0, 52.2] and 14.3 [10.2, 18.4] months for all patients, respectively. However, for patients with advanced stage survival was 25.8 [21.1, 32.2] for OS and 10.2 [8.37, 5.0] for PFS.

5.2 Influence of clinico-pathologic characteristics on clinical outcomes of platinum-based chemotherapy

5.2.1 Overall population

5.2.1.1 Response

Response was better in squamous cell carcinoma (p=0.0342; RR=1.30; $CI_{95\%}$=1.02-1.66; Table S1), I, II and IIIA stage (p=0.0053; RR=1.41; $CI_{95\%}$=1.11-1.79; Table S1), and tumor resection (p=0.0004; RR=1.56; $CI_{95\%}$=1.22-1.99; Table S1).

5.2.1.2 Survival

The clinical factors associated with OS were: female ($p_{log\text{-}rank}$=0.0082; 85.4 vs 27.0 months; Table S2; Figure S1), squamous cell carcinoma ($p_{log\text{-}rank}$=0.0021; 59.4 vs 26.1 months; Table S2; Figure S2), I, II and IIIA stage ($p_{log\text{-}rank}$<0.001; 85.4 vs 25.8 months; Table S2; Figure S3), paclitaxel chemotherapy agent ($p_{log\text{-}rank}$<0.001; Table S2; Figure S4) and tumor resection ($p_{log\text{-}rank}$<0.001; 114.0 vs 26.1 months; Table S2; Figure S5). Similarly, median PFS was better in female ($p_{log\text{-}rank}$ =0.0418; 19.6 vs 11.0 months; Table S3; Figure S6), squamous cell carcinoma ($p_{log\text{-}rank}$=0.0034; 27.3 vs 11.4 months; Table S3; Figure S7), I, II and IIIA stage ($p_{log\text{-}rank}$<0.001; 44.0 vs 10.2 months; Table S3; Figure S8), paclitaxel as chemotherapy agent ($p_{log\text{-}rank}$<0.001; Table S3; Figure S9), tumor resection ($p_{log\text{-}rank}$<0.001; 197.0 vs 17.0 months; Table S3; Figure S10). A trend towards better PFS was showed in those patients who received concomitant or concurrent radiotherapy but it was not statistically significant ($p_{log\text{-}rank}$=0.0566; 23.2 vs 11.2 months; Table S3; Figure S11).

5.2.2 Subgroup analysis

5.2.2.1 Response

In the subgroup of native *EGFR* response was better in I, II and IIIA stage (p=0.0291; RR=1.63; $CI_{95\%}$=1.05-2.53; Table S4), and tumor resection (p=0.0057; RR=1.74; $CI_{95\%}$=1.12-2.70; Table S4). However, in patients with mutations in *EGFR* no association with clinical or demographic characteristics was found (Table S5).

5.2.2.2 Survival

For patients with native *EGFR* median OS was higher in females ($p_{log\text{-}rank}$=0.0336; 47.4 vs 24.5 months; Table S6; Figure S12), personal history of cancer ($p_{log\text{-}rank}$=0.0319; 47.4 vs 25.4 months; Table S6; Figre S13), I, II and IIIA stage ($p_{log\text{-}rank}$=0.0113; 59.4 vs 23.1 months; Table S6; Figure S14), paclitaxel chemotherapy agent ($p_{log\text{-}rank}$<0.001; Table S6; Figure S15) and surgery as first course of treatment ($p_{log\text{-}rank}$=0.0018; 73.9 vs 23.1 months; Table S6; Figure S16). Similarly, median PFS was higher in females ($p_{log\text{-}rank}$=0.017; 18.7 vs 8.5 months; Table S7; Figure S17), I, II and IIIA stage ($p_{log\text{-}rank}$=0.014; 23.3 vs 10.0 months; Table S7; Figure S18), paclitaxel chemotherapy agent ($p_{log\text{-}rank}$<0.001; Table S7; Figure S19) and surgery as first course of treatment ($p_{log\text{-}rank}$<0.001; 40.8 vs 9.2 months; Table S7; Figure S20).

In the subgroup of patients with mutations in *EGFR* median OS was higher in females ($p_{log\text{-}rank}$=0.005; 52.2 vs 21.1 months; Table S8; Figure S21) and tumor resection ($p_{log\text{-}rank}$=0.023; 126.4 vs 24.2 months; Table S8; Figure S22). Similarly, median PFS was better in surgery as first course of treatment ($p_{log\text{-}rank}$=0.042; 49.4 vs 5.8 months; Table S9; Figure S23). A trend to higher risk of progression was also showed in male ($p_{log\text{-}rank}$=0.221; 6.9 vs 6.0 months; Table S9; Figure S24) and stage I, II, IIIA ($p_{log\text{-}rank}$=0.866; 6.5 vs 4.2 months; Table S9; Figure S25).

5.3 Genotype distribution

Genotype frequencies were in agreement with the values expected under the Hardy-Weinberg equilibrium model. Linkage disequilibrium values D' and r^2 are shown in Table S10. No linkage disequilibrium was revealed in any case. The frequencies of these polymorphisms were compared with those reported by HapMap-CEU (Table S11). Significant differences were found for *ERCC2* rs13181, *ERCC1* rs1799793, *MDM2* rs1690927, *MTHFR* rs1801133 and *XRCC1* rs25487 gene polymorphisms.

5.4 Influence of gene polymorphisms on clinical outcomes of platinum-based chemotherapy

5.4.1 Overall population

5.4.1.1 Response

XRCC1 rs25487 was associated with response. Patients carrying the GG genotype showed significantly better ORR compared to those with AG/AA genotypes (dominant model) (p=0.0343; RR=1.29; $CI_{95\%}$=1.02, 1.63; Table S12). A trend towards better ORR was showed for those patients with *ERCC1* rs3212986-GG genotype, but it was not statistically significant (dominant model) (p=0.0825; RR=1.24; $CI_{95\%}$=0.97, 1.58; Table S12). Logistic regression model adjusted by resection revealed that *XRCC1* rs25487-GG genotype and *ERCC1* rs3212986-GG were independently associated with response ($p_{likelihood\ ratio\ test}=8.663 \cdot 10^{-7}$; Table 2).

5.4.1.2 Survival

5.4.1.2.1 Overall survival

Kaplan-Meier curve for *MDM2* rs1690924 polymorphism showed a trend to higher risk of death for GG genotype, but this was not statistically significant (recessive model) ($p_{log\text{-}rank}$=0.086; Table S13) (Figure S26). Patients with GG genotype showed a median OS of 17.5

months ($CI_{95\%}$= 15.2-Not reached (NR)), whereas for AG and AA genotypes was 43.1 ($CI_{95\%}$=30.7-85.4) and 32.2 ($CI_{95\%}$=24.5-73.9) months, respectively. Patients with CC genotype for *XRCC1* rs1799782 polymorphism showed a trend to higher risk of death compared to those carrying the T-allele, but this was not statistically significant either (dominant model) (p=0.0777; HR=1.88; $CI_{95\%}$=0.93-3.79; Table S13). The Figure S27 shows the Kaplan-Meier curve in accordance to *XRCC1* rs1799782-T allele ($p_{log\text{-}rank}$=0.073). Median OS was 30.0 months ($CI_{95\%}$=25.4-41.8) for CC genotype, whereas for CT genotype the median OS was 85.4 months ($CI_{95\%}$=48.4-NR). For the TT genotype, the survival median values exceeded the survival time of the further observation.

Clinical (gender, histology, tumor stage, chemotherapy reagents, surgery) and genetic variables (*MDM2* rs1690924 and *XRCC1* rs1799782) associated with overall survival were analyzed by multivariate analysis to identify potential confounding effects. Multivariate Cox regression adjusted by gender, tumor histology, chemotherapy agents and surgery revealed that *MDM2* rs1690924 gene polymorphism was associated to OS ($p_{likelihood\ ratio\ test}=3.391\cdot10^{-13}$; Table 3).

5.4.1.2.2 Progression-free survival

No associations were demonstrated in the bivariate analysis between polymorphisms and PFS. However, the A-allele for *MTR* rs1805087 (recessive model) ($p_{log\text{-}rank}$=0.106; Table S14) (Figure S28) and the AA genotype for *SLC19A1* rs1051266 polymorphisms (recessive model) ($p_{log\text{-}rank}$=0.053 in patients treated with pemetrexed and $p_{log\text{-}rank}$=0.127 in all the patients; Table S14) (Figures S29 and S30) presented a trend to higher risk to progression. Patients with A-allele for MTR rs1805087 polymorphism showed a median PFS of 12.9 months ($CI_{95\%}$=10.2-17.6) versus GG genotype, which revealed a median PFS of 82.3 months ($CI_{95\%}$=82.3-NR). On the other hand, patients treated with pemetrexed showed a median PFS for *SLC19A1* rs1051266-AA genotype of 6.05 months ($CI_{95\%}$=4.27-NR), whereas for G-allele the median PFS was 9.07 months ($CI_{95\%}$=5.2-16.9). In all the patients median PFS for *SLC19A1* rs1051266-AA genotype was 10.1 months ($CI_{95\%}$=7.0-20.4), whereas for G-allele the median PFS was 15.0 months ($CI_{95\%}$=10.9-23.2).

Clinical (gender, histology, tumor stage, chemotherapy reagents, surgery) and genetic variables (*MTR* rs1805087 and *SLC19A1* rs1051266) associated with progression-free survival were analyzed by multivariate analysis to identify potential confounding effects. Multivariate Cox regression model showed that gender, surgery and concomitant or concurrent radiotherapy were the clinical variables with impact on PFS. *MTR* rs1805087 and *SLC19A1* rs1051266 were significantly associated with PFS ($p_{likelihood\ ratio\ test}= 2.22\cdot10^{-16}$; Table 4)

5.4.2 Subgroup analysis

5.4.2.1 Response

In the *EGFR* native subgroup, carriers of the GG genotype for *XRCC1* rs25487 showed significantly better ORR compared to those with AG/AA genotypes, as described in the overall population (p=0.0379; RR=1.51; $CI_{95\%}$=1.02, 2.23; Table S15). Logistic regression model adjusted by tumor stage revealed that *XRCC1* rs25487-GG genotype was the only independent factor associated to response in NSCLC patients with native *EGFR* (Table 5). However, in the subgroup of patients with mutations in *EGFR,* no association with gene polymorphisms was found (Table S16).

5.4.2.2 Survival

5.4.2.2.1 Overall survival

Patients with native *EGFR* and carriers of the AA genotype for *SLC19A1* rs1051266 gene polymorphism were in higher risk of death compared to those with G-allele (p=0.044; HR=1.86;

$CI_{95\%}$=1.02-3.41; Table S17). Kaplan-Meier curves for OS according to G-allele for *SLC19A1* rs1051266 gene polymorphism are showed in Figure S31 ($p_{log\text{-}rank}$=0.041). Median OS for AA carriers was 24.5 ($CI_{95\%}$=17.1-32.4) months, whereas for G-allele was 32.2 ($CI_{95\%}$=23.1-85.6). A trend to higher risk of death was also showed in those patients with native *EGFR* and GG genotype for the *MDM2* rs1690924 gene polymorphism, which was in accordance with the results described in the overall population (p=0.283; HR=1.58; $CI_{95\%}$=0.69-3.62; Table S17). Kaplan-Meier curves for OS according to A-allele for *MDM2* rs1690924 gene polymorphism are showed in Figure S32 ($p_{log\text{-}rank}$=0.279). Median OS for patients carrying AA genotype was 25.4 ($CI_{95\%}$=23.1-NR) months, whereas for AG and GG genotypes was 30.7 ($CI_{95\%}$=15.5-64.7) and 15.2 ($CI_{95\%}$=9.6-NR) months, respectively. Multivariate Cox regression adjusted by gender, chemotherapy reagents and tumor resection showed that *MDM2* rs1690924 gene polymorphism was the only independent factor associated to OS in NSCLC patients with native *EGFR* ($p_{likelihood\ ratio\ test}$= $2.885 \cdot 10^{-07}$; Table 5), which is in accordance with the results described in the overall population.

In the subgroup of patients with mutations in *EGFR,* the bivariate analysis reported that carriers of the *ERCC1* rs3212986- T-allele showed higher risk of death, compared to those with GG genotype (dominant model) (p=0.0179; HR=4.68; $CI_{95\%}$=1.30-16.76; Table S18). Kaplan-Meier curves for OS according to T-allele for *ERCC1* rs3212986 gene polymorphism are showed in Figure S33 ($p_{log\text{-}rank}$=0.012). Patients with GG genotype showed a median OS of 30.0 months ($CI_{95\%}$= 30.-NR), whereas for T-allele was 20.9 ($CI_{95\%}$=20.9-NR). *MTR* rs1805087 was also associated with OS. Patients carrying the AA genotype presented longer OS compared to those carrying the G-allele (p=0.050; HR=3.35; $CI_{95\%}$=1.00-11.23; Table S18). Kaplan-Meier curves for OS according to *MTR* rs1805087 gene polymorphism are showed in Figure S34 ($p_{log\text{-}rank}$=0.039). Patients with AA genotype showed a median OS of 52.2 months ($CI_{95\%}$= 21.1-NR), whereas for G-allele was 20.9 ($CI_{95\%}$=15.8-NR). Similarly, the *SLC19A* rs1051266-G allele was associated with higher risk of death compared to those with AA genotype (p=0.039; HR=9.10; $CI_{95\%}$=1.11-74.46; Table S18). Kaplan-Meier curves for OS according to G-allele for *SLC19A* rs1051266 gene polymorphism are showed in Figure S35 ($p_{log\text{-}rank}$=0.015). Patients with AA genotype showed a median OS of 105.9 months ($CI_{95\%}$= 30.0-NR), whereas for genotype AG was 23.9 ($CI_{95\%}$=18.3-NR), and for GG was 21.1 ($CI_{95\%}$=15.8-NR). However, no association with gene polymorphisms was found in the multivariate Cox regression model.

5.4.2.2.2 Progression-free survival

In the subgroup with native *EGFR*, patients carrying the AC/AA genotype for *MTHFR* rs1801131 gene polymorphism were in higher risk of progression, compared to those with CC (recessive model) (p=0.050; HR=2.80; $CI_{95\%}$=1.00-7.86; Table S19). Kaplan-Meier curves for PFS according to A-allele for *MTHFR* rs1801131 gene polymorphism are showed in Figure S36 ($p_{log\text{-}rank}$=0.041). Patients with CC genotype showed a median PFS of 34.5 months ($CI_{95\%}$= 15.5-NR), whereas for genotype AC was 10.6 ($CI_{95\%}$=6.0-19.6), and for AA was 9.2 ($CI_{95\%}$=7.0-17.1). *MTR* rs1805087 also showed a trend towards higher progression, but this was not statistically significant. In fact, patients carrying the A-allele presented higher risk of progression compared to those carrying the GG genotype (p=0.279; HR=2.23; $CI_{95\%}$=0.52-9.57; Table S19). Kaplan-Meier curves for PFS according to A-allele for *MTR* rs1805087 gene polymorphism are showed in Figure S37 ($p_{log\text{-}rank}$=0.269). Patients with GG genotype showed a median PFS of 82.3 months ($CI_{95\%}$= 2.3-NR), whereas for A-allele genotype was 10.3 ($CI_{95\%}$=7.7-16.8). A multivariate Cox regression model adjusted by gender tumor stage, chemotherapy reagents and tumor resection was used to evaluate the impact of gene polymorphisms on PFS (Table 5). *MTHFR* rs1801131 and *MTR* rs1805087 gene polymorphism were significantly associated with PFS ($p_{likelihood\ ratio\ test}$= $7.972 \cdot 10^{-09}$).

ERCC1 rs3212986 gene polymorphism showed influence on PFS only in NSCLC patients with mutations in *EGFR* (Table S20). In fact, patients carrying the GT/TT genotypes presented higher

risk of progression compared to those carrying the GG genotype (p=0.0267; HR=6.31; $CI_{95\%}$=1.24-32.16; Table S20). Kaplan-Meier curves for PFS according to T-allele for *ERCC1* rs3212986 gene polymorphism is showed in Figure S38 ($p_{log\text{-}rank}$=0.013). Patients with GG genotype showed a median PFS of 15.6 months ($CI_{95\%}$=6.9-NR), whereas for GT and TT genotypes, the median PFS was 5.7 ($CI_{95\%}$=3.7-NR) and 4.7 ($CI_{95\%}$=4.7-NR) months, respectively. A trend to higher risk of progression was also showed in those patients with mutations in *EGFR* and TT genotype for the *ERCC2* rs13181 gene polymorphism, but this was not statistically significant (dominant model) (p=0.0758; HR=2.96; $CI_{95\%}$=0.89-9.79; Table S20). Kaplan-Meier curves for OS according to G-allele for *ERCC2* rs13181 gene polymorphism are showed in Figure S39 ($p_{log\text{-}rank}$=0.063). Patients with G-allele genotype showed a median PFS of 6.5 ($CI_{95\%}$=5.8-NR), whereas for TT genotype was 4.9 months ($CI_{95\%}$= 4.2-NR). Kaplan-Meier curve for *MTR* rs1805087 polymorphism showed a trend to higher risk of progression for AA genotype, but this was not statistically significant ($p_{log\text{-}rank}$=0.883; Table S20) (Figure S40). Patients with AA genotype showed a median OS of 5.8 months ($CI_{95\%}$= 4.2-NR), whereas for G-allele was 6.0 ($CI_{95\%}$=5.6-NR). Multivariate Cox regression adjusted by gender and tumor stage showed that *ERCC1* rs3212986, *ERCC2* rs13181 and *MTR* rs1805087 gene polymorphisms were associated to PFS in NSCLC patients with mutations in *EGFR* ($p_{likelihood\ ratio\ test}$= 0.0003848; Table 5).

6 DISCUSSION

Chemotherapy based on platinum compounds, used as the standard treatment for NSCLC patients with *EGFR* wild-type, and also as second line in mutated *EGFR* patients, presents poor clinical outcomes [5]. The inter-individual variability described among patients with the same clinic-pathologic characteristics may be partly explained by genetic factors. Polymorphisms involved in DNA repair pathway and folate metabolism have been proposed as leading cause of these inter-individual differences. In this study, a total of 141 NSCLC patients, treated with platinum compounds in combination with a third-generation drug, were enrolled to evaluate the potential role of *ERCC1, ERCC2, XRCC1, MDM2, MTHFR, MTR* and *SLC19A1* gene polymorphisms in chemotherapy clinical outcomes. *ERCC1* rs3212986-GG genotype was associated with better response in our patients (Table 2). Previous studies have reported similar results. In Asian population, two studies with 115 and 163 patients have reported worse ORR to platinum-based chemotherapy in patients carrying the T-allele (OR=0.23; $CI_{95\%}$=0.10, 0.57 for AC/AA vs CC and OR=0.44; $CI_{95\%}$=0.27, 0.74 for T vs G allele, respectively) [56, 57]. Remarkably, this SNP is located in the 3'-adjacent gene *CAST* (CD3ε-associated signal transducer) and causes an amino acid change in the CAST protein. CAST is an RNA polymerase I-specific subunit and has a role in the activation of transcription [58]. Additionally, it has been suggested that polymerase I may exerts a crucial effect on sensing for DNA damage, indicating a role of CAST in DNA repair [59]. However, whether this polymorphism alters the functional activity of CAST is as yet unknown.

The XRCC1 protein is the key component of the BER pathway, which interacts with DNA polymerase-beta, DNA ligase III and PARP (poly ADP-ribose polymerase), repairing the damaged DNA strand [60]. Although its functional effect has not been well known, XRCC1 rs25487, occurs in the PARP binding domain of *XRCC1* gene, may affect complex assembly, and reduce DNA repair efficiency [61]. In our patients, the GG genotype for *XRCC1* rs25487 was associated with better ORR compared to those with AG/AA genotypes (Table 2). This result is in consonance with a recent meta-analysis, which evaluated 13 studies and 1334 cases from Asian population (OR=2.05; $CI_{95\%}$=1.62, 2.60; I^2=26%; $P_{heterogeneity}$=0.18; GG vs AG/AA) [30]. However, no significant association had previously been reported in Caucasian patients [62-67]. We also found that patients carrying *MDM2* rs1690924-GG genotype were in higher risk to death (Table 3). To date, the functional function of this SNPs is unknown and no other studies have found association between this polymorphism and OS [33]. However, the GG genotype for

MDM2 rs1690924 has been related to lower gastrointestinal toxicity (OR=2.32; $CI_{95\%}$=1.30, 4.14 for AG vs AA) in 663 Chinese NSCLC patients [33]. In our patients, we also observed that those carrying the *MTR* rs1805087-A allele or *SLC19A1* rs1051266-AA genotype were associated with higher risk of progression (Table 4). The *MTR* gene plays a crucial function on folate metabolism. Although the direct functional impact of this polymorphism has not been established, there is some evidence that this may be an activating polymorphism; some studies have reported increased enzymatic activity in individuals with GG genotype [68]. Furthermore, individuals with GG genotype have showed lower frequency of CpG island hypermethylation in tumor suppressor genes [69].To date, no other studies have explored the effect of *MTR* rs1805087 on PFS in NSCLC patients treated with platinum-based. However, two studies in 101 IIIB/IV and 465 I-IV NSCLC patients failed to find an association with response (OR=0.66; $CI_{95\%}$=0.23, 1.89 for AG/GG vs AA) and OS (HR=0.99; $CI_{95\%}$=0.23, 1.89 for AG/GG vs AA) [35, 36]. The influence of *SLC19A1* rs1051266 on clinical outcomes of platinum-based chemotherapy has also been explored, showing no association [37-41]. Our patients with *SLC19A1* rs1051266-AA genotype previously showed a greater risk of gastrointestinal toxicity to platinum compounds [70]. However, there is no scientific evidence available of effect of this SNP on protein expression or activity.

In order to confirm if these results were the same considering positive and negative EGFR patients in different groups, a stratified analysis based on *EGFR* status was performed. The same results as described for all the patients were found in patients with native *EGFR*. Moreover, an association between PFS and *MTHFR* rs1801131 polymorphism was observed, which had not been shown for all the patients. In fact, patients with *MTHFR* rs1801131-A allele were in higher risk of progression compared to those carrying the CC genotype. This result is in consonance with a previous study in 1004 Chinese stage III/IV NSCLC patients that reported lower ORR (OR=1.52; $CI_{95\%}$=1.04, 2.23 for AC vs AA) and PFS (p=0.03) in patients carrying *MTHFR* rs1801131-AA genotype [71]. In patients with mutant *EGFR,* no significant association was found for response and OS, but the *ERCC1* rs3212986-T allele, *ERCC2* rs13181-TT and *MTR* rs1805087-AA genotype was associated with shorter PFS (Table 5). The *ERCC1* rs3212986 and *ERCC2* rs13181 polymorphisms were not associated with PFS when all patients were considered in the analysis. The influence of *ERCC1* rs3212986 on survival in NSCLC patients was also reported in previous studies showing shorter OS and PFS in patients carrying the T-allele [56, 72-75]. Our results also show the negative effect of *ERCC2* rs13181-T allele on PFS, as previously described in 353 Asian stage IIIB/IV NSCLC patients (HR=1.54; $CI_{95\%}$=1.03, 2.29 for GT/TT vs GG) [76]. However, a previous meta-analysis including 22 studies/3240 patients reported no association between ORR (OR=0.93; $CI_{95\%}$=0.78, 1.12; I^2=0.0%; $P_{heterogeneity}$=0.707; CC/AC vs AA) and PFS (HR=1.08; $CI_{95\%}$=0.93, 1.25; I^2=28%; $P_{heterogeneity}$=0.187; AA/AG vs GG) [28]. Intriguingly, our results showed a protective effect for advance stage both in native and mutant *EGFR* (Table 5). However, this effect was not directly because of the stage, but a consequence of the small subgroups sizes, and especially the bad outcome of three patients with stage IIIA, probably affected with a more aggressive tumor, who could not be treated with surgery due to neoadjuvant chemotherapy unresponsiveness. These patients experienced a very rapid progression in less than 4 months, thereby unbalancing the PFS median in the group of early stage (I-IIIA).

The effect of *ERCC1* rs11615 on chemotherapy outcomes in NSCLC patients has been extensively investigated, with conflicting results. Some studies have reported better ORR, OS and PFS in patients carrying the CC genotype [56, 63, 72, 74, 77-84], whereas others have described higher ORR, OS and PFS in patients with T-allele [57, 66, 74, 84-89]. In our study, *ERCC1* rs11615 showed no association neither with response or survival, which is in consonance with the two meta-analysis which have analyzed the compiled results of most of the other studies [28, 90]. Previous results for *XRCC1* rs1799782 have reported better ORR for T-allele in Asian population, but not in Caucasian patients [66, 91-96]. A recent meta-analysis, which involved 11

studies and compiled 1329 cases, has reported similar results in Asian population (OR=0.38; $CI_{95\%}$=0.30, 0.48; I^2=0%; $P_{heterogeneity}$=0.830; CT/TT vs CC) [30]. No associations between OS, PFS and *XRCC1* rs1799782 SNPs have been found [66, 96-102]. In our study, this polymorphism, along with *ERCC2* rs1799793, was not associated with platinum based chemotherapy outcomes. This lack of association of *ERCC2* rs1799793 with ORR and PFS is in consonance with a previous meta-analysis including 22 studies/3240 patients [28], which reported no association between ORR (OR=0.87; $CI_{95\%}$=0.70, 1.08; I^2=44.8%; $P_{heterogeneity}$=0.041; AA/AG vs GG) or PFS (HR=1.15; $CI_{95\%}$=0.93, 1.41; I^2=24.2%; $P_{heterogeneity}$=0.266; AA/AG vs GG). However, this polymorphisms has been associated with OS in several studies [63, 76, 103, 104]. In our study, the *MDM2* rs1470383 gene polymorphism was not associated with clinical outcomes of platinum-based chemotherapy. To date, the association between this SNP and response has not been evaluated, being only related to hematological toxicity to chemotherapy in an Asian study with 663 Chinese NSCLC patients (OR=4.10; $CI_{95\%}$=1.73, 9.71); no association with OS and PFS was found [33]. *MTHFR* rs1801133 were not associated with clinical outcomes of platinum-based chemotherapy in our patients. Nevertheless, a meta-analysis compiling data from 3 studies and 147 patients, both in Asian and Caucasian populations, has also shown better response in individuals with *MTHFR* rs1801133-TT genotype (OR=1.72; $CI_{95\%}$=1.01, 2.93; I^2=16%; $P_{heterogeneity}$=0.31; TT vs CT/CC) [34]. Additionally, the *MTHFR* rs1801133-TT genotype has also been associated with higher OS (p=0.026) and PFS (p=0.012) in 208 Italian stage IIIB/IV NSCLC patients [38].

The frequencies of these SNPs in our population were compared with those reported by Hapmap-CEU. Significant differences were found for *ERCC2* rs13181, *ERCC1* rs1799793, *MDM2* rs1690927, *MTHFR* rs1801133 and *XRCC1* rs25487 gene polymorphisms. The reason of these differences may be because these polymorphisms are associated with risk of NSCLC, as it is described in several meta-analysis [34, 105-107].

The limitations of our study include a limited sample size and a considerable number of genetic and clinical covariates that may have reduced the group sizes for some comparisons, which may be responsible of the lack of association between some polymorphisms. However, the recruitment of a single hospital cohort, following the same therapeutic protocols by the same team of oncologists ensured its homogeneity and reliability of the response variables. All patients diagnosed during the period of study were recruited, ensuring the representativeness of the sample. Despite the limited sample size, the effects observed in these patients were evident. Further studies in larger cohorts will be necessary to confirm the predictive value of some of the biomarkers, particularly *ERCC1, XRCC1, MDM2, MTR* and *SLC19A1* gene polymorphisms in the management of NSCLC patients.

In summary, these results showed that *ERCC1* rs3212986, *XRCC1* rs25487, *MDM2* rs1690924, *MTR* rs1805087, *SLC19A1* rs1051266 gene polymorphisms may significantly act as predictive factors in NSCLC patients treated with platinum-based chemotherapy.

7 CONCLUSIONS

Our results suggest that *ERCC1* rs3212986-GG and *XRCC1* rs25487-GG genotypes are associated with better ORR. NSCLC patients carrying the MDM2 rs1690924-GG genotype were in higher risk of death. The *MTR* rs1805087-A alleles and the *SLC19A1* rs1051266-AA genotype were associated with greater risk of progression. No association between *ERCC1* rs11615, *ERCC2* rs13181, *ERCC2* rs1799793, *XRCC1* rs1799782, *MDM2* rs1470383, *MTHFR* rs1801131, *MTHFR* rs1801133 and clinical outcomes of platinum-based chemotherapy was found in our patients.

8 AUTHOR CONTRIBUTIONS

Cristina Pérez-Ramírez revised the bibliography and wrote the protocol for this study and acted as the primary lead in the conception, design and implementation of the project, data

collection, molecular analysis, statistical analysis and interpretation, as well as all aspects of the development and writing of the article and responses to internal and external reviewers.

Marisa Cañadas-Garre, PhD, supervised the planning of the study protocol and contributed to the conception, design and implementation of the project, statistical analysis and interpretation as well as all aspects of the development and writing of the article and responses to internal and external reviewers.

Ahmed Alnatsha performed literature review, molecular analysis and interpretation, clinical data collection and interpretation and collaborated in drafting the manuscript.

Eduardo Villar, MD, PhD, performed molecular and pathological analysis and interpretation, clinical data collection and interpretation and collaborated in drafting the manuscript.

Javier Valdivia Bautista, MD participated in the clinical data collection and interpretation, and provided clinical guidance and feedback for this study and critical review of the manuscript.

María José Faus-Dáder, PhD and **Miguel Ángel Calleja-Hernández**, PhD, participated in article selection, study quality assessment, critical review of the manuscript and contributed to revisions and responses to internal and external reviewers.

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10 FIGURE LEGENDS

Figure S1. A) Kaplan-Meier curve for overall survival according to gender in 141 NSCLC patients. B) Kaplan-Meier curve for overall survival according to histology in 141 NSCLC patients. C) Kaplan-Meier curve for overall survival according to tumor stage in in 141 NSCLC patients. D) Kaplan-Meier curve for overall survival according to chemotherapy reagents in 141 NSCLC patients. E) Kaplan-Meier curve for overall survival according to surgery in 141 NSCLC patients.

Figure S2. A) Kaplan-Meier curve for progression-free survival according to gender in 141 NSCLC patients. B) Kaplan-Meier curve for progression-free survival according to histology in 141 NSCLC patients. C) Kaplan-Meier curve for progression-free survival according to tumor stage in 141 NSCLC patients. D) Kaplan-Meier curve for progression-free survival according to chemotherapy reagents in 141 NSCLC patients. E) Kaplan-Meier curve for progression-free survival according to surgery in 141 NSCLC patients. F) Kaplan-Meier curve for progression-free survival according to concomitant or concurrent radiotherapy in 141 NSCLC patients.

Figure S3. A) Kaplan-Meier curve for overall survival according to gender in patients with native *EGFR*. B) Kaplan-Meier curve for overall survival according to personal history of cancer in patients with native *EGFR*. C) Kaplan-Meier curve for overall survival according to tumor stage in patients with native *EGFR*. D) Kaplan-Meier curve for overall survival according to chemotherapy reagents in patients with native *EGFR*. E) Kaplan-Meier curve for overall survival according to surgery in patients with native *EGFR*.

Figure S4. A) Kaplan-Meier curve for progression-free survival according to gender in patients with native *EGFR*. B) Kaplan-Meier curve for progression-free survival according to tumor stage in patients with native *EGFR*. C) Kaplan-Meier curve for progression-free survival according to chemotherapy reagents in patients with native *EGFR.* D) Kaplan-Meier curve for progression-free survival according to surgery in patients with native *EGFR.*

Figure S5. A) Kaplan-Meier curve for overall survival according to gender in patients with mutations in *EGFR*. B) Kaplan-Meier curve for overall survival according to surgery in patients with mutations in *EGFR.*

Figure S6. A) Kaplan-Meier curve for progression-free survival according to surgery in patients with mutations in *EGFR*. B) Kaplan-Meier curve for progression-free survival according to gender in patients with mutations in *EGFR*. C) Kaplan-Meier curve for progression-free survival according to tumor stage in patients with mutations in *EGFR.*

Figure S7. A) Kaplan-Meier curve for overall survival according to A-allele for *MDM2* rs1690924 gene polymorphism in 141 NSCLC patients. B) Kaplan-Meier curve for overall survival according to T-allele of *XRCC1* rs1799782 gene polymorphism in 141 NSCLC patients.

Figure S8. A) Kaplan-Meier curve for progression-free survival according to A-allele of *MTR* rs1805087 gene polymorphism in 141 NSCLC patients. B) Kaplan-Meier curve for progression-free survival according to G-allele of *SLC19A1* rs1051266 gene polymorphism in patients

treated with pemetrexed. C) Kaplan-Meier curve for progression-free survival according to G-allele of *SLC19A1* rs1051266 gene polymorphism in 141 NSCLC patients.

Figure S9. A) Kaplan-Meier curve for overall survival according to G-allele of *SLC19A1* rs1051266 gene polymorphism in patients with native *EGFR*. B) Kaplan-Meier curve for overall survival according to A-allele of *MDM2* rs1690924 gene polymorphism in patients with native *EGFR.*

Figure S10. A) Kaplan-Meier curve for overall survival according to T-allele of *ERCC1* rs3212986 gene polymorphism in patients with mutations in *EGFR*. B) Kaplan-Meier curve for overall survival according to G-allele of *MTR* rs1805087 gene polymorphism in patients with mutations in *EGFR.* C) Kaplan-Meier curve for overall survival according to G-allele of *SLC19A1* rs1051266 gene polymorphism in patients with mutations in *EGFR.*

Figure S11. A) Kaplan-Meier curve for progression-free survival according to A-allele of *MTHFR* rs1801131 gene polymorphism in patients with native *EGFR.* B) Kaplan-Meier curve for progression-free survival according to A-allele *MTR* rs1805087 gene polymorphism in patients with native *EGFR.*

Figure S12. A) Kaplan-Meier curve for progression-free survival according to T-allele of *ERCC1* rs3212986 gene polymorphism in patients with mutations in *EGFR.* B) Kaplan-Meier curve for progression-free survival according to G-allele of *ERCC2* rs13181 gene polymorphism in patients with mutations in *EGFR.* C) Kaplan-Meier curve for progression-free survival according to G-allele of *MTR* rs1805087 gene polymorphism in patients with mutations in *EGFR.*