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Review

Predictive Role of Repair Enzymes in the Efficacy of Cisplatin Combinations in Pancreatic and Lung Cancer

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Abstract. *Platinum combinations are the mainstay of treatment for non-small cell lung cancer (NSCLC), while for pancreatic cancer platinum combinations are being given to good-performance status patients. These platinum combinations consist of cis- or carboplatin with gemcitabine, while, for non-squamous NSCLC and mesothelioma, of pemetrexed. The combination of gemcitabine and cisplatin is based on gemcitabine-induced increased formation and retention of DNA–platinum adducts, which can be explained by a decrease of excision repair cross-complementing group-1 (ERCC1)-mediated DNA repair. In these patients, survival and response is prolonged when ERCC1 has a low protein or mRNA expression. A low expression of ribonucleotide reductase (RR) is related to a better treatment outcome after both gemcitabine and gemcitabine-platinum combinations. For pemetrexed combinations, ERCC1 expression was not related to survival. For both NSCLC and pancreatic cancer, polymorphisms in ERCC1 (C118T) and Xeroderma pigmentosum group D (XPD) (A751C) were related to survival. In currently ongoing and future prospective studies, patients should be selected based on their DNA repair status, but it still has to be determined whether this should be by immunohistochemistry, mRNA expression, or a polymorphism.*

Non-small cell lung cancer (NSCLC) and pancreatic ductal adenocarcinoma (PDAC) account for approximately 85% of lung and pancreatic cancer, respectively, and have a 5-year survival of less than 5% (1). Platinum combinations are standard therapy for both NSCLC and malignant pleural mesothelioma (MPM) but are still experimental in PDAC (2). Pemetrexed-platinum therapy is standard for MPM and non-squamous NSCLC, while for squamous NSCLC, gemcitabine-cisplatin is often used (3). The combination of raltitrexed and cisplatin was as similarly active in MPM as pemetrexed–cisplatin or pemetrexed–carboplatin (4).

All combinations are based on the assumption that in the combination, DNA damage is more extensive and repair is inhibited. Among the various DNA repair systems, the nucleotide excision repair (NER) system seems the major system involved in the repair of DNA–platinum adducts (5, 6), although the mismatch repair system also plays a role for cisplatin and carboplatin, but not for oxaliplatin (7). Several proteins are involved in the recognition of DNA damage, unwinding, subsequent excision of the damaged nucleotides (AG and GG Pt–adducts), and insertion of new deoxynucleoside triphosphates (dNTP) in the DNA. These include transcription factor II H (TFIIH), the xeroderma pigmentosum group enzymes XPD, XPC, ERCC1/XPF, DNA polymerases δ and ε, and ligase 1 (Figure 1). Single nucleotide polymorphisms (SNP) in any of these genes may affect the repair capacity and contribute to individual variations in chemotherapy response. Hence these repair systems have been extensively been investigated as an explanation for the interaction of gemcitabine with cisplatin (8, 9). Such pharmacogenetic studies may allow the variation in how individual patients respond to medicines to be reduced by tailoring therapies to their genetic profile (10).

Pre-clinical Basis for The Interaction of Gemcitabine and Cisplatin

The molecular basis for the interaction between cisplatin and gemcitabine was initially investigated in cell lines of ovarian, head and neck, and NSCLC (11-13). In these model systems,

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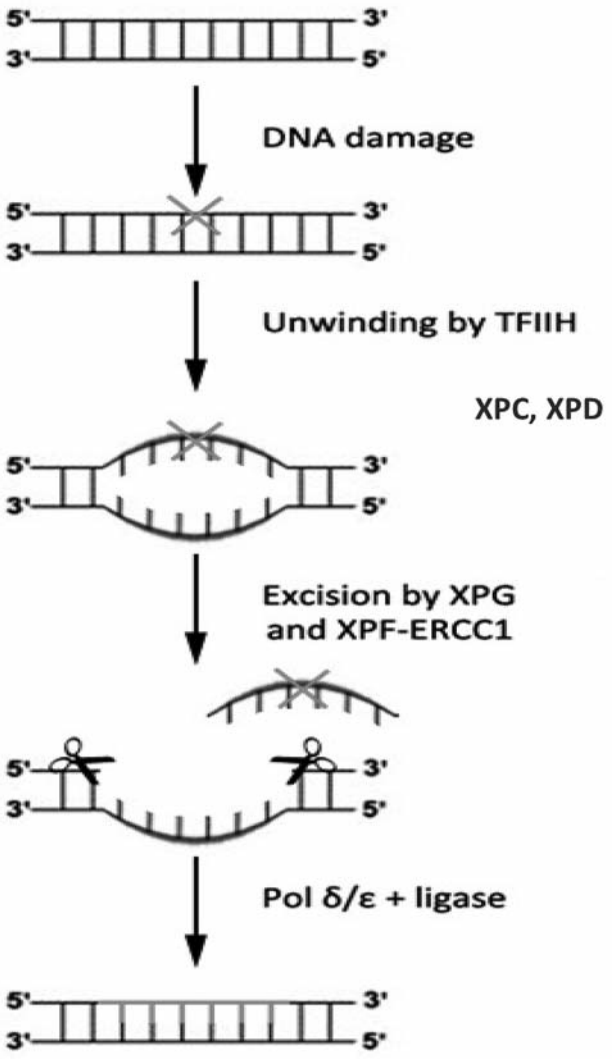

Figure 1. *Schematic action of the nucleotide excision repair (NER) pathway. In NER, after DNA damage is recognized, the DNA helix is unwound by transcription factor II H (TFIIH), which is stabilized by Xeroderma pigmentosum group D (XPD). The TFIIH subunits of XPD and XPB/C act as a helicase and ATPase, respectively. Next incisions are made both up- and downstream of the lesion by XPG and XPF-excision repair cross-complementing group 1 (ERCC1). XPG acts as an endonuclease which cuts DNA damage on the 3' side while the XPF-ERCC1 heterodimeric protein cuts on the 5' side. The dual incision leads to the removal of a ssDNA with a single-strand gap of 25-30 nucleotides. DNA polymerase then uses the undamaged single-stranded DNA as a template to synthesize a short complementary sequence, followed by ligation via DNA ligase to complete NER and form a double-stranded DNA. Partially modified from (6).*

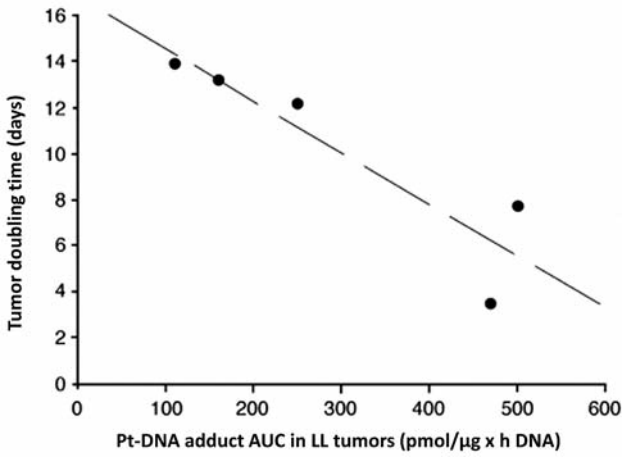

Figure 2. *Correlation between Pt–DNA adduct formation and retention (expressed as area under the curve of adducts) and sensitivity of Lewis lung tumors to various gemcitabine–cisplatin treatments. The treatment with the highest extent of adducts was also the most effective treatment (Van Moorsel et al. (15), with permission).*

we demonstrated one of the most pronounced synergisms between two drugs, with combination indices (CI) of 0.001 and less (11). The combination was also more than additive in *in vivo* tumors generated from these cell lines (12-14); the most active schedule consisted of every 3-day gemcitabine schedule (four times), combined with cisplatin only on the first day. Both gemcitabine-preceding-cisplatin and cisplatin preceding gemcitabine were very active, although the gemcitabine preceding cisplatin became the most widely used schedule. Detailed analysis of the molecular interaction demonstrated that gemcitabine increased the formation of cisplatin–DNA adducts (13-16), both *in vitro* and *in vivo*. A detailed analysis in murine Lewis lung with NSCLC showed that Pt–DNA adduct levels (especially Pt–GG), evaluated as the areas under the concentration–time curves of Pt–DNA in the tumors, were associated with reduced doubling-time of tumors treated with the combination and this was related to the antitumor effect (Figure 2). *In vitro* it was also demonstrated that cisplatin increased the incorporation of gemcitabine into DNA (13). Most likely this increased incorporation was responsible for a structural change in the DNA, allowing more DNA adducts to be formed; this change might also affect inhibition of DNA repair. The extent of ERCC-mediated DNA repair in the gemcitabine–cisplatin combination was correlated with the extent of synergism (17). Selvakumaran *et al.* demonstrated in an *in vivo* system with *ERCC1* antisense transfectants that ERCC1 is essential for *in vivo* repair of DNA platinum adducts (18).

For the repair of DNA damage, DNA polymerases require a sufficient supply of dNTP which are provided by the action of ribonucleotide reductase (RR), which has two subunits RRM1 and RRM2. Inhibition of RR by gemcitabine reduces the concentration of dATP, dGTP and dCTP (19), this decrease hampers the repair of both AG and GG adducts. Hence RR expression is likely to play a role in the repair of Pt–DNA adducts, as outlined below.

Genetic and epigenetic alterations, such as gene mutations, amplification, deletions, polymorphic status, or altered gene/protein expression have been shown to be correlated with drug responses, including of gemcitabine and platinum analogs (6, 8, 20). Among the possible predictive or prognostic factors of survival benefit to a specific treatment, germline polymorphisms have been identified as an attractive target, specifically for advanced cancer, since their analysis can be more easily performed compared to tumor mutational analysis and gene expression arrays (21). Therefore, in the following, we mainly focused on gene expression alterations or polymorphic status of the genes involved in DNA repair systems and drug metabolism.

ERCC1 Expression and Repair of Pt–DNA Adducts in Lung Cancer

Since its introduction into the clinic, the cisplatin–gemcitabine combination is still considered a standard regimen for the treatment of advanced NSCLC (22). In a randomized four-arm phase III study, no difference was observed in time-to-progression (TTP), and overall survival (OS) between paclitaxel–cisplatin, docetaxel–cisplatin, paclitaxel–carboplatin, and gemcitabine–cisplatin combinations (2) and in a three-arm study between paclitaxel-cisplatin, gemcitabine-cisplatin or paclitaxel-gemcitabine (23). Because of better tolerance, the gemcitabine–cisplatin (or carboplatin) regimen is still considered as the standard regimen, although pemetrexed has replaced gemcitabine for non-squamous NSCLC based on a phase III study (3).

In an initial study by Lord *et al*. (24), it was demonstrated that patients with NSCLC with a low *ERCC1* mRNA expression (as determined by PCR) had increased survival compared to those with high *ERCC1* expression. In subsequent analyses both mRNA expression by PCR and immunohistochemistry were used to determine the expression of ERCC1. Although some antibodies against ERCC1 recognized another protein (25-27), a meta-analysis from 12 studies and 836 patients clearly demonstrated that low levels of *ERCC1* mRNA or protein expression were associated with a longer survival [odds ratio (OR)=0.77, 95% confidence interval (CI)=0.47-1.07, $p<0.00001$] and a superior major response rate (OR=0.48, 95% CI=0.35-0.64, $p<0.0001$) (28). An even stronger correlation was observed when different parameters were combined. Ceppi *et al*. (29) demonstrated that a low expression of ERCC1 or RRM1 each were associated with a longer survival for patients treated with gemcitabine–cisplatin (ERCC1 $p=0.0032$; RRM1 $p=0.039$) but when the two were combined (both low), the survival benefit increased and was more significant ($p=0.0023$); similar results were observed by Bepler *et al*. (30). This information was recently applied in order to select patients on the treatment arm most likely to be sensitive to these combinations (31); from 275 eligible patients, those with a low RRM1/ERCC1 expression were randomized for the gemcitabine–carboplatin combination, those with a high RRM1 and low ERCC1 for docetaxel–cisplatin, and those with a high RRM1 and high ERCC1 for docetaxel–vinorelbine. No statistically significant differences were observed between the experimental and control arms regarding progression-free survival (6.1 *vs*. 6.9 months) and overall survival (11.0 *vs*. 11.3 months). However, all patients who received the same treatment and had a low expression of ERCC1/RRM1 had better progression-free survival (8.1 months) in the control group, compared to the experimental arm (5.0 months). Another major conclusion of this study was that measurement of protein expression was feasible and very reproducible.

Both immunohistochemistry and PCR analysis may have problems regarding their use in large groups of patients, since antibodies have to be validated (which is not always done properly) and may change in time (25- 27), while sufficient RNA cannot always be isolated from tumor samples, albeit the technology to isolate sufficient and high quality RNA from paraffin-embedded tissues has improved considerably in the last decade. We compared immunohistochemistry, PCR and genetic polymorphisms for several biomarkers for their potential in patients with MPM (32). Indeed immunohistochemistry proved to be too variable to draw firm conclusions on the ERCC1 expression in this cohort of patients. In NSCLC, we found a high expression in cytoplasm, which was associated with a longer survival, a finding which seems counterintuitive, but this might be explained by the localization (Figure 3a).

An *in vitro* analysis demonstrated that the C/T SNP at codon 118 of the *ERCC1* gene could have an influence on mRNA and protein levels (33), while some clinical data support a possible correlation of this SNP with survival in advanced NSCLC being treated with platinum-based chemotherapy (34, 35). Therefore we analyzed this polymorphism in our patients. In this group of patients, we observed that the *ERCC1* C118T SNP was associated with a larger number of responding patients (Figure 3b). Expression of *ERCC1* was also related to the C118T SNP; in cytoplasm of tumor cells of patients with a TT genotype, there was a low intensity (0-1), in those with a CT genotype a two-fold higher intensity (0-2). In the nuclei of tumor cells of patients with a TT genotype, there was a high intensity (0-6), but in those with a CT genotype a lower intensity (0-4) (Figure 3c-d). Another intriguing finding in this cohort of patients was the association of an *XPD* polymorphism (A751C lys-gln) with longer survival of these patients (Figure 4). For the pemetrexed–carboplatin combination A751 gln-gln was associated with a shorter survival (36). Another polymorphisms of XPD (Asp312asn) was associated with longer survival in patients treated with a carboplatin–taxane

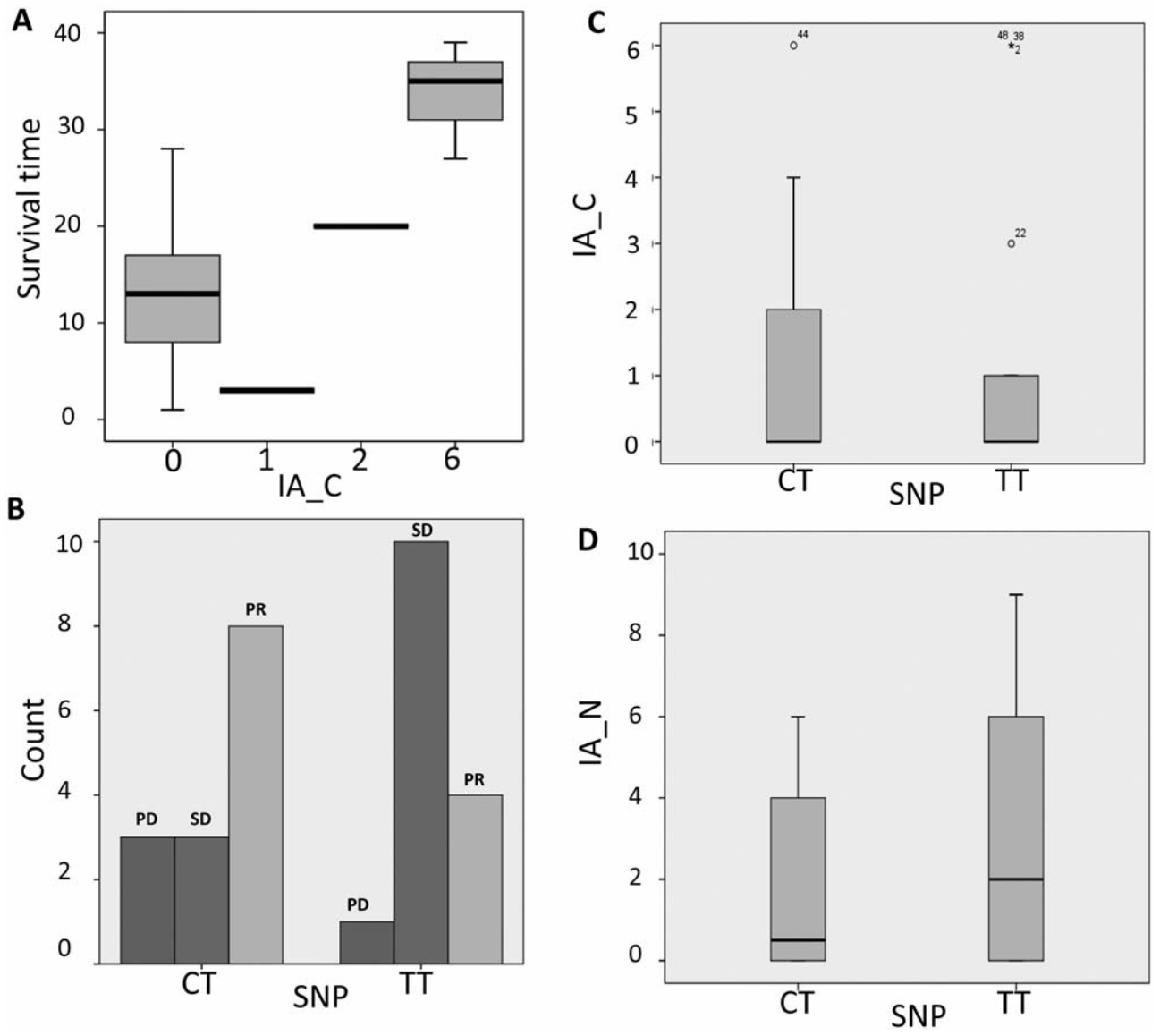

Figure 3. *Association of excision repair cross-complementing group 1 (ERCC1) expression with C118T polymorphism, cellular distribution and survival of patients with non-small cell lung cancer (NSCLC) treated with gemcitabine and cisplatin. Forty-six patients (13 female/34 male; 22 adenocarcinoma, 8 squamous and 17 large cell carcinoma) were analyzed (overall median survival=14.6 months). Expression of ERCC1 is given on a scale from 0 (low to absent) to 6 (very strong) and was performed as described earlier (26). SNP analysis was performed with quantitative polymerase chain reaction as described earlier (43). A: Association of cytoplasmic (IA_C) expression of ERCC1 with survival time (months). A total of 47 patients were analyzed, with 26 patients in the low expression group (Pearson correlation 0.656, p<0.0001). B: Association of the C118T genotype with progressive disease (PD), stable disease (SD) and partial response (PR); count represents the number of patients. C: Association of cytoplasmic ERCC1 staining (IA_C) with the CT and TT genotypes of C118T. D: Association of nuclear staining (IA_N) with the C118T genotype.*

combination (37), as well as NSCLC (38). It can be concluded that in NSCLC, ERCC1 and RRM1 expression and *ERCC1* and *XPD* polymorphisms may be associated with response to cisplatin therapy. However, a recent meta-analysis concluded that the predictive value of *ERCC1* and *XPD* polymorphisms in patients with advanced NSCLC receiving platinum-based chemotherapy may both be important (39). The discrepancies observed among the studies may be due to differential methods, treatment heterogeneity, and relatively small sample size.

Triggered by the analysis of various DNA repair systems in the process of DNA platinum adduct repair, we analyzed these parameters in MPM, also treated with a platinum combination. The standard treatment for MPM is a

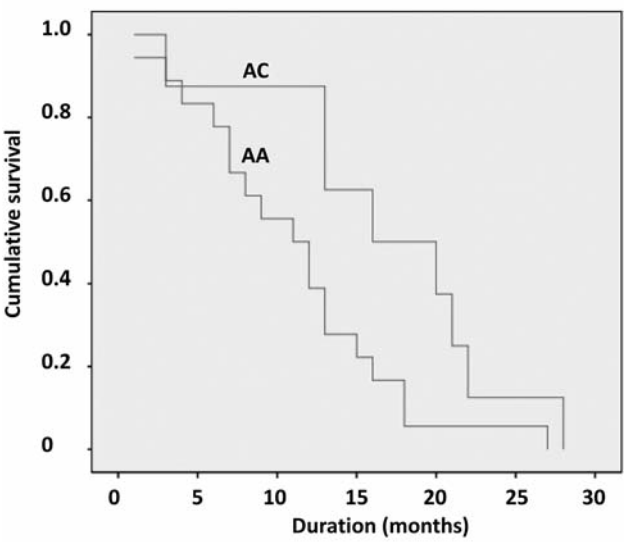

Figure 4. *Association of the Xeroderma pigmentosum group D (XPD) A751C (Lys/Gln) genotype (67% AA and 33 AT genotype; Hardy-Weinberg equilibrium 0.147) with survival of patients with non-small cell lung cancer (NSCLC) treated with gemcitabine and cisplatin.*

combination of the antifolate pemetrexed and cisplatin or carboplatin. Pemetrexed is a multi-targeting antifolate, which not only inhibits its major target thymidylate synthase, but also dihydrofolate reductase, as well *de novo* purine nucleotide synthesis. Inhibition of thymidylate synthase will lead to a decrease in dTTP, which will affect DNA repair, but differently compared to inhibition of RRM1. In this study, a low thymidylate synthase expression was associated with a longer survival (18.0 months) compared to those patients who has a high expression (9 months, p=0.022) (32). In the same cohort of patients, ERCC1 expression by immunohistochemistry failed to show any significant association, with an overall survival of 12 months for the low expression and 18 for the higher expression groups.

The Role of DNA Repair in PDAC

Standard treatment in PDAC is gemcitabine, which replaced 5-fluorouracil (5FU) in the late 1990s. From all combinations which have been investigated, the platinum combinations seemed most promising, in a setting either with cisplatin or oxaliplatin (40, 41). The combination treatment of FOLFIRINOX (5FU with leucovorin, irinotecan and oxaliplatin) produced the best results in patients with a good performance status who were able to tolerate this potentially toxic regimen. An alternative, the oral 5FU formulation S-1 with cisplatin produced a longer survival in patients with the *ERCC1* C118T genotype (CT and TT; p=0.030) (42). In an analysis of 122 patients, treatment with gemcitabine-based polychemotherapy, the TT genotype was associated with a 13.3-month median survival (95% CI=9.7-17.0) compared to CC+TT (11.8 months, 95% CI=10.4-13.4; p=0.44) (43). For XPD, an association between polymorphisms was found for both the *XPD* asp312Asn and *XPD* lys-751gln. The Asn-Asn genotype was associated with a shorter overall survival (11.2 months, 95% CI=10.9-15.7 months) compared to the Asp-Asp phenotype (15.1 months, 95% CI=10.4-19.4 months; p=0.010). Similarly the *XPD* GLn751Gln had a shorter survival of 10.3 (95% CI=4.0-16.5) months compared to the LysLys + Lys+Gln cohort (13.3 months, 95% CI=10.9-15.7; p=0.003). Survival of those with *XPD* Gln751Gln was shorter compared to those with other genotypes. In a larger cohort, 247 patients were treated with multiple drug combinations (PEXG: gemcitabine-cisplatin + epirubicin-xeloda; PDXG: gemcitabine-cisplatin + docetaxel-xeloda; EC-Gem-Cap: gemcitabine-cisplatin (i.a.) + Epirubicin (i.a.)-xeloda (i.a.) or gemcitabine alone (90 patients). In this polychemotherapy schedule, the *XPD* Gln751Gln conferred a poor survival (Figure 5a) (44). In this study, the genetic polymorphisms were also investigated for a functional association assuming that a decreased repair ability is related with the formation of more Pt–DNA adducts; indeed, white blood cells with the specific genotype (Lys751Lys) have a lower repair capacity compared with cells with Gln751Gln); this formation of Pt–DNA adducts was enhanced by gemcitabine in the Lys751Lys cohort (Figure 5b). Hence, the analysis of the polymorphism by a simple blood test offers an innovative tool for optimizing palliative chemotherapy in patients with advanced PDAC.

Conclusion

The efficacy of platinum combinations is dependent on the formation of platinum adducts. The extent of their formation and retention are controlled by DNA repair enzymes, but the contribution of each enzyme may be different for various diseases and combinations. For example, in a recent study in 19 head and neck cancer cell lines, the sensitivity to cisplatin was associated with DNA adduct formation and retention but not with the expression of any repair enzyme or other potential markers of resistance, such as drug transporters, which were shown to play a role in cisplatin resistance (45). It seems that in NSCLC treated with gemcitabine–cisplatin combinations, ERCC1 expression is related to efficacy, but this does not hold true for pemetrexed combinations in MPM.

Another major problem is the method used to determine the expression of the mRNA the protein. Although immunohistochemistry is a sensitive and versatile method for determination of protein expression, it is largely empirical, and the outcome mainly depends on which antibody is being used and also on the pathologist's expertise. A major

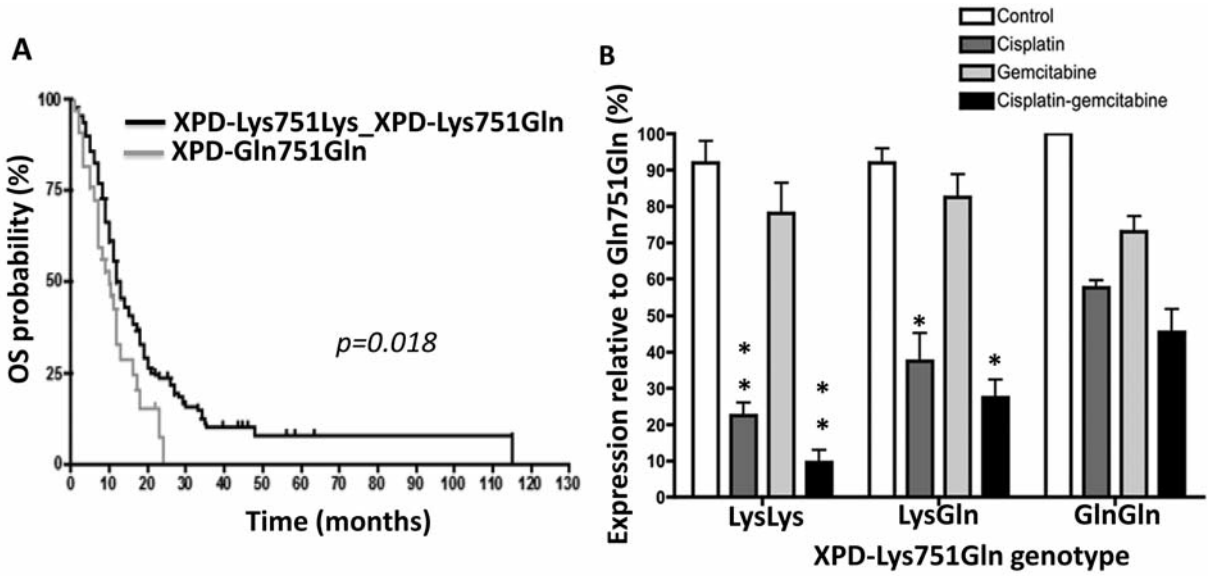

Figure 5. *A: Association of the Xeroderma pigmentosum group D (XPD) A751C genotype with overall survival of patients with pancreatic ductal adenocarcinoma (PDAC) (213 patients with XPD Lys751Lys, or XPD Lys751Gln and 33 with XPD Gln751Gln, median overall survival 13.0 (11.4-14.6) and 7.0 (4.0-10.0) months) treated with gemcitabine- and cisplatin-containing poly-chemotherapy regimen (44). From (44) with permission. B: White blood samples from five volunteers per group A751A, A751C and C751C were tested for their DNA repair ability after exposure to 1 μM gemcitabine, 200 μM cisplatin or the combination for 24 h, after which a target sequence of β-globin was amplified with extra-long PCR (XL-PCR) as described earlier (44). The extent of DNA repair ability was based on the reduction of PCR amplification of the target sequence. Data are means ±SD. Significantly different from *XPD Gln751Gln, **XPD Gln751Gln, or XPD Lys751Gln.*

disadvantage of protein expression is the potential lack of specificity of antibodies (26, 27), potentially leading to incorrect expression data. Another problem is the cellular distribution of the protein, as shown for the cytoplasmic and nuclear staining (Figure 3). Hence new antibodies should be characterized thoroughly, preferably in model systems lacking or having high expression. Several studies have used the quantitative RT-PCR technique, but mRNA expression can differ from protein expression. mRNA isolation was considered a problem, but nowadays mRNA can be isolated in reliable quantities, even from paraffin-embedded tissues. The disadvantage is the lack of pathological confirmation of the sample, although RNA can be isolated from tumor-enriched parts, or one can use laser microdissection. Therefore, optimization and standardization of these two modalities with appropriate controls, which can be used for inter-laboratory validation, are essential before larger prospective investigations in homogeneously treated patients, which can address the same pharmacogenetic question.

From the same sample, DNA can be isolated for SNP analysis. Although SNP analysis using DNA is much more reliable, most data are not strong enough (small groups) to use in prospective studies. These studies are essential to select those patients who are likely to respond to the standard treatment and to select patients who are eligible for therapy with tyrosine kinase inhibitors such as erlotinib and gefitinib for patients with activating mutations for *EGFR*, or with crizotinib for patients with ALK expression (46). Since many of these tyrosine kinase inhibitors are synergistic with DNA-targeted therapy, such as gemcitabine–cisplatin or pemetrexed–cisplatin, pre-treatment analysis of gene expression or SNP will help select the treatment most likely to be effective.

Further investigations are needed to evaluate these emerging biomarkers, as well as to identify and select the optimal patient populations that will benefit from specific treatments. Together with the standardized techniques for sample collection and processing, larger and uniformly treated populations, and integration with functional data are necessary in order to validate the best markers for personalized treatment of patients.

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