Clinical and Molecular Determinants of Survival in Pancreatic Cancer Patients Treated with Second-line Chemotherapy: Results of an Italian/Swiss Multicenter Survey

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Abstract. *Background: Increased knowledge about the treatment of pancreatic cancer has influenced the management of locally advanced and metastatic disease. Nonetheless, prognosis remains dismal (24%, 1-year survival). The impact on overall survival (OS) of second-line therapy has not been clarified and the use of platinum salts and/or fluoropyrimidines is hotly debated. It is the hope that future treatment can be tailored to predict chemosensitivity in order to improve outcomes in patients with locally advanced and metastatic pancreatic cancer. Since DNA-damaging agents could be one therapeutic option, a retrospective multicenter study was performed to evaluate the efficacy of salvage treatment with the hypothesis that levels of the DNA repair gene excision repair cross complementing 1 (ERCC1) could influence OS. Patients and Methods: In a population of 160 patients treated with fluoropyrimidine-based second-line chemotherapy, expression levels of ERCC1 were determined by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). In 108 patients with locally advanced and metastatic pancreatic cancer treated with either fluoropyrimidines and platinum salts (group A=58) or fluoropyrimidines alone (group B=50), ERCC1 levels were correlated with OS, time to progression and response to chemotherapy. Results: Median survival was significantly higher in group A with low ERCC1 levels [11.9 versus 9.9 months; p=<0.05] (median follow-up 24 months). Moreover in the same group, a trend towards longer time to progression was observed. No differences in OS were observed when ERCC1 was studied (low versus high) in patients not treated with platinum salts. On multivariate analysis of pretreatment prognostic factors, ERCC1 emerged as an independent predictive factor for OS. Conclusion: The results of this study indicate that ERCC1 may predict survival in pancreatic cancer patients treated by platinum and fluoropyrimidine as second- line chemotherapy.*

Metastatic pancreatic adenocarcinoma is a disease with a extremely poor prognosis. In 2008, an estimated 37,680 new cases of pancreatic cancer were diagnosed in the USA, with an almost identical death rate of 34,290 (1). Without treatment, median survival for patients (pts) with advanced-stage disease ranges from 3 to 4 months, whereas in these receiving chemotherapy with single-agent gemcitabine, median survival between 4.9 and 7.2 months has been reported in randomized phase III studies (2, 3). The role of salvage treatment after failure of first-line gemcitabine-containing chemotherapy remains controversial. The National Comprehensive Cancer Network guidelines for pancreatic adenocarcinoma currently recommend second-line chemotherapeutic treatment after gemcitabine failure in selected pts using fluoropyrimidine alone or fluoropyrimidine- based regimens (4). Results from different trials show prolongation of median survival by approximately 2.6 months with the use of chemotherapy (2.3 *versus* 4.9 months). Clinical/molecular predictive factors are needed in order to decide which therapeutic regimen may be most effective (5, 6). The aim of this multi-institutional study was to evaluate the efficacy of fluoropyrimidine-based second-

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line chemotherapy in all pts and to determine the relationship between the expression status of excision repair cross-complementation group 1 (ERCC1) and chemosensitivity in those pts receiving platinum salts and fluoropyrimidines. In addition to the predictive role of *ERCC1* this study explored its prognostic role in pts treated only with fluoropyrimidines.

Patients and Methods

Patients and samples. Clinical data of 160 pts treated between June 1997 to February 2006 were retrospectively collected from the databases of 11 medical oncology departments in Italy and Switzerland. Inclusion criteria for this survey were histologically documented metastatic, gemcitabine-resistant or -refractory pancreatic adenocarcinoma. All patients received second-line treatment immediately after first-line progression, and measurable disease with an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 2 or less was required. Archival primary tumor specimens from each pts were retrieved from the participating centers.

Clinical response criteria. Prior to second-line treatment, all pts underwent disease assessment consisting of a complete history, physical examination, appropriate imaging studies (chest X-rays, abdomen and pelvis computed tomography scan), complete blood count and biochemistry. Weight, ECOG PS and tumor markers (CEA and CA19.9) were also registered as reported in clinical charts. Blood cell counts and biochemistry were repeated at the beginning of each treatment cycle. Sixteen different salvage regimens were administered consisting of monotherapy with fluoropyrimidines in 43% of cases and combinations of platinum-salts/fluoropyrimidines in 51%. Fluoropyrimidine combinations with bevacizumab, irinotecan and mitomycin C were administered in the remaining 6% (Table I).

Table I. *Patient characteristics*.

	Number	%
Total no. of patients	160	100
Gender		
Male	99	61.8
Female	61	38.2
Age (years)		
Median	62	
Range	34-78	
Performance status (ECOG)		
0	58	36
1	54	34
2	48	30
Second-line treatments (tested *ERCC1* population *versus* whole study population)		
Fluoropyrimidines	50/17	32/11
Oxaliplatin/5-FU CVI	37/8	23/5
Cisplatin/Mit-C	5/4	3/2
Cisplatin/5-FU CVI	13/7	9/4
Carboplatin/5-FU CVI	3/6	2/3
Fluoropyrimidine combinations	0/10	0/6
No. of involved sites		
1	85	53
2	75	47
Organ involved (multiple involved)		
Peritoneum	16	21
Lymph node	20	27
Liver	26	35
Lung	8	11
Other	5	6

Within the context of this retrospective study, response was assessed using Response Criteria in Solid Tumors (RECIST) (7) and by re-evaluation of known sites of disease by physical examination, imaging after every two to three months of treatment, or as clinically indicated. Patients who received at least two months of treatment were considered assessable for response. Follow-up evaluations were recorded for PS, weight, toxicity, complete blood counts, serum creatinine and blood urea nitrogen levels.

Gene expression analysis by immunohistochemical (IHC) staining and real-time quantitative polymerase chain reaction (RT-QPCR). Two approaches were used to analyze excision repair cross-complementation group 1: Tumors were examined for excision repair cross-complementation group 1 expression by immunohistochemistry (IHC) of the gene product and mRNA expression using RT-QPCR. Slides of tumor samples stained with hematoxylin and eosin were reviewed by a pathologist who had no knowledge of patient clinical data. Core tissue biopsy specimens (2 mm in diameter) in duplicate were obtained from individual paraffin-embedded samples (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips, Seoul, Korea). Sections (4 μm) were cut from each tissue array block, placed on slides and deparaffinized, and dehydrated. Immunohistochemistry was performed according to international guidelines as previously described for other gene products (8). In brief, antigen retrieval was performed by microwaving 4 μm sections in 0.01 M citrate buffer (pH 6.0) for 15 min at 650 W. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 15 min. After incubating sections with blocking solution for 10 min, sections were incubated with mouse monoclonal anti-ERCC1 antibody clone 8F1 (1/50 dilution; NeoMarkers, Fremont, CA, USA) at 4˚C for 12 h followed by biotinylated secondary mouse monoclonal anti-ERCC1 antibody clone 8F1 at room temperature for 10 min, and then streptavidin horseradish peroxidase for 10 min. Staining was carried out with diaminobenzidine chromogen, and counter staining with Mayer's hematoxylin. Blocking solution, secondary antibody, streptavidin horseradish peroxidase, and diaminobenzidine chromogen were all from the Cap-Plus Kit (Zymed Laboratories, San Francisco, CA, USA). Stromal cells surrounding the tumor area served as internal positive controls. Staining was graded for intensity of staining (1, weak; 2, moderate; 3, strong) and percentage of cells stained (1, 0% to <10%; 2, 10% to <50%; 3, 50% to 100%). Staining for excision repair cross-complementation group 1 was considered to be positive when tumor cells showed nuclear reactivity and both scores (H-score) were two or above (Figure 1).

For PCR, a laser capture microdissection procedure (Palm Microlaser, Oberlensheim, Germany) was used in order to have a minimum of 80% of tumor tissue. After standard tissue sample deparaffinization and lysate preparation, RNA was then extracted with phenol–chloroform–isoamyl alcohol, precipitated with isopropanol in the presence of glycogen and sodium acetate, and

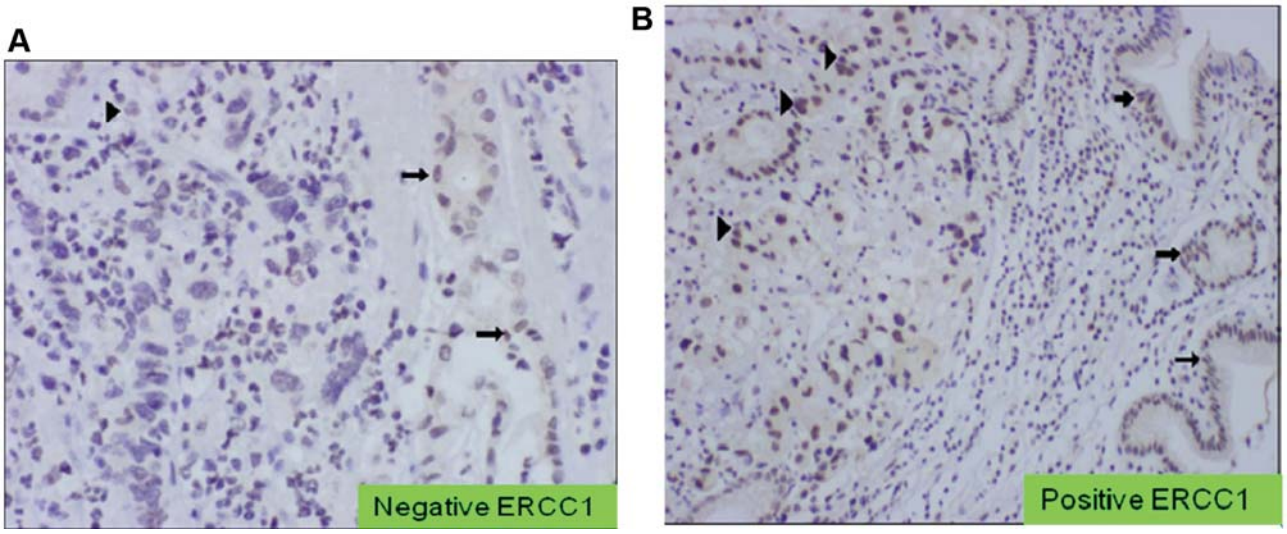

Figure 1. *Representative examples of ERCC1 immunostaining. A: H score <2; B: H score ≥2. Original magnification, ×400.*

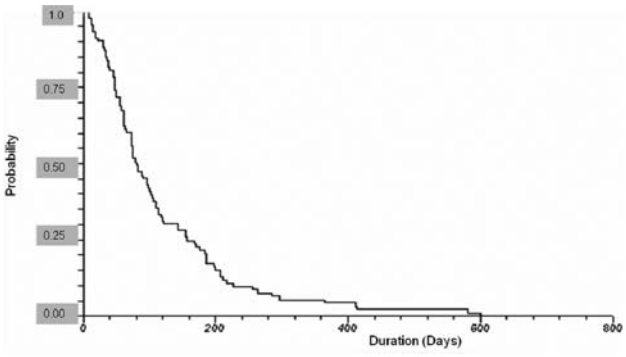

Figure 2. *Kaplan-Meier progression-free survival (all evaluable patients) after second-line therapy.*

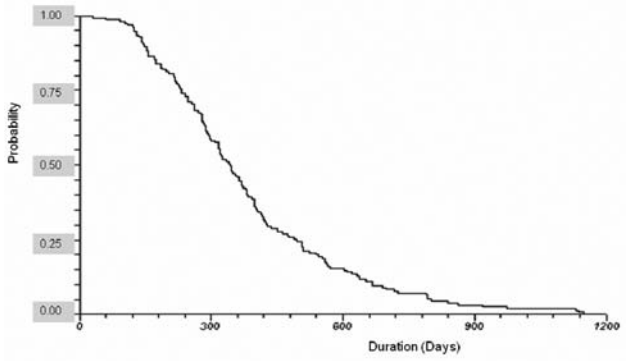

Figure 3. *Kaplan-Meier cumulative overall survival (all evaluable patients).*

resuspended in diethyl pyrocarbonate water (Ambion Inc., Austin, TX, USA). DNAse I (Ambion Inc.) was used to avoid DNA contamination. Complementary DNA (cDNA) was synthesized using Maloney murine leukemia virus retrotranscriptase enzyme. Template cDNA was added to Taqman Universal Master Mix (AB, Applied Biosystems, Foster City, CA, USA) in a 12.5-μl reaction with specific primer and probe for *ERCC1*. Quantification of gene expression was performed using the ABI Prism 7900HT Sequence Detection System (AB). Relative gene expression quantification was calculated according to the comparative cycle threshold method using β-actin (*ACTB*) as an endogenous control.

Statistical analyses. QPCR analyses yield values expressed as ratios between two absolute measurements (gene of interest/internal reference gene). The χ^2 maximal method of Halpern (9) was adapted to determine the cut-off value that best dichotomized into those with negative expression and those with positive expression of *ERCC1*. The proportion was compared with the Fisher's exact test. The Kaplan–Meier method was used to calculate overall survival (OS) and time to progression (TTP) respectively from diagnosis to death and from the end of second-line treatment to disease progression. The Cox proportional hazards model was used to examine the prognostic and predictive value of gene expression levels together with other pretreatment factors, including age, sex, PS, presence of visceral metastases, number of disease sites and histology. Factors that showed individual prognostic value in the univariate model were examined in a multivariate model. Spearman correlation coefficient was performed to assess associations between mRNA expression levels and congruity rate with IHC excision repair cross-complementation group 1. All analyses were carried out with the SPSS software package, version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics and overall results. Baseline characteristics are shown in Table I. The median age was 62 years (range 34-78 years) and 62% of patients were male.

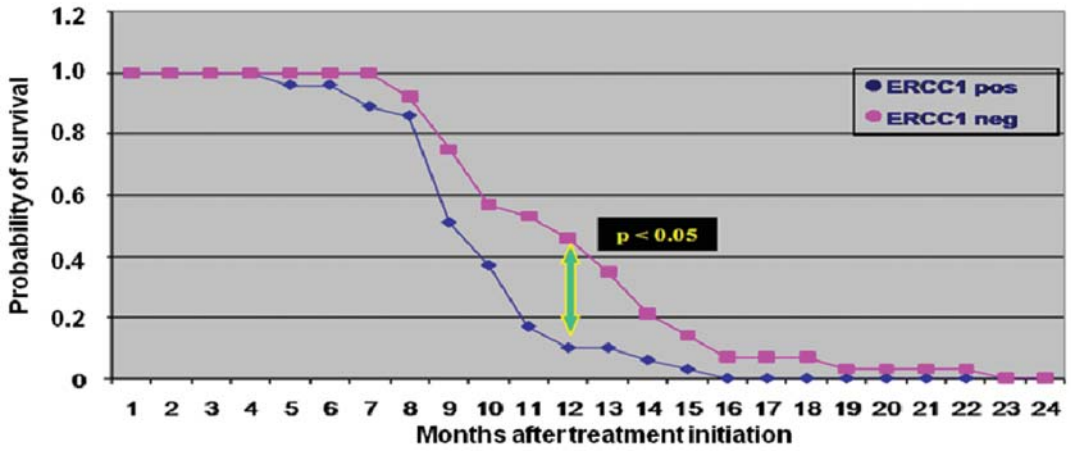

Figure 4. *Overall survival according ERCC1 status (platinum-treated patients).*

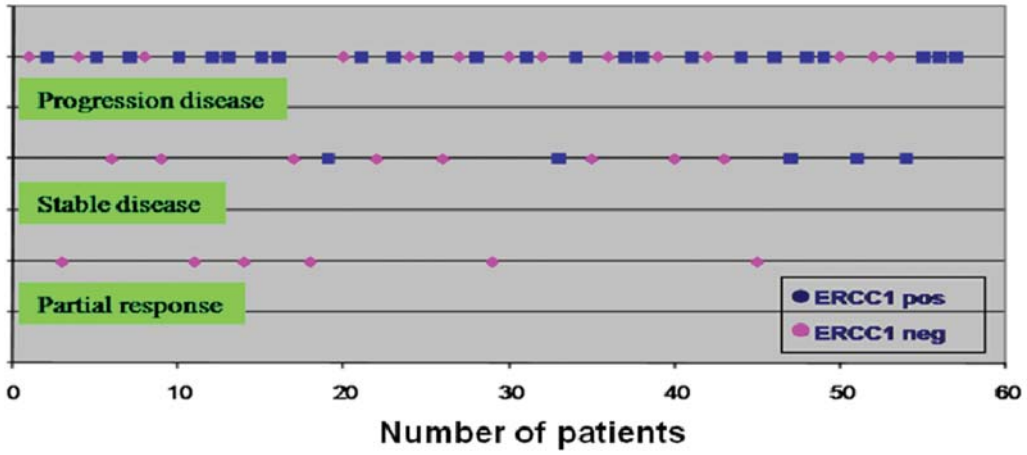

Figure 5. *Overall response rates according to ERCC1 status.*

One-hundred andtwo patients (64%) had a PS of one or two and 58 (36%) had a PS of zero. Multiple visceral metastases (lung, peritoneal, lymph node, liver or bone) were present in over 80% of cases. Second-line chemotherapy produced partial responses (cPR) in 16 (10%) and stable disease (cSD) in 40 pts (25%). The overall tumor growth control (PR+SD) rate was 35%. The median TTP was 2.65 months (Figure 2).

Multivariate analysis revealed that the most important prognostic factor for TTP was PS, as pts with a PS of 2 at the beginning of second-line therapy had a significantly worse outcome than those with PS=0-1 (second-line TTP: 78 days *versus* 48 days, $p \leq 0.05$). Baseline CA19-9 and number of metastatic sites were not independent prognostic factors for better second-line TTP. Pts who had responded to first-line gemcitabine were more likely to respond or achieve cSD after second-line treatment, with a TTP of 2.6 *versus* 1.6 months ($p \leq 0.05$). With a median follow-up of 24 months, the OS for all evaluable pts was 11.5 months and 1-year survival was 45% (Figure 3). At the time of analysis, no patients were alive.

Gene expression levels. ERCC1 IHC and mRNA expression were assessed in all 58 samples from pts treated with platinum salts (group A) and in 50 pts treated with fluoropyrimidines only (group B); 52 pts were not studied due to inadequate histological material for molecular studies. The median *ERCC1* mRNA expression relative to the housekeeping gene (*ACTB*) was 8.9 (range 3.3-20.2). The results for ERCC1 IHC and mRNA expression were significant and consistent with a congruity rate of 93% (Spearman r=0.168, $p=0.034$).

Gene expression levels and survival. Fifty-seven pts were treated with platinum salts; with a relative *ERCC1* expression cut-off of 9, 28 patients were classified as being *ERCC1*-negative and 29 as exhibiting positive *ERCC1* expression. OS was significantly longer for *ERCC1*-negative pts (11.9 months, 95% confidence intervals CI, 8.65-17.69 months) than for those with positive ERCC1 levels (9.9 months, 95% CI 6.13-12.77 months, $p \leq 0.05$) (Figure 4).

No differences in OS were observed when *ERCC1* was studied (negative *versus* positive) in pts not treated with platinum salts. In both groups of pts, univariate analysis was performed on prognostic groups based on six predefined factors. Of these variables, PS≥2 and high *ERCC1* mRNA levels were associated with an adverse prognosis in group A; in group B, only PS≥2 was correlated with a poor outcome. Histology, sex, age, presence of visceral metastases, number of disease sites, histology and lactate dehydrogenase level did not have a significant effect on survival in either group. When the two variables that emerged as significant in univariate analyses were included in a multivariate regression model, *ERCC1* levels and PS were identified as independent prognostic markers in group A. A worse outcome was observed in pts with PS=2 (hazard ratio 3.88, 95% CI 1.28-8.21, $p=0.001$) and in pts with high levels of *ERCC1* (hazard ratio 2.72, 95% CI 1.23-7.40, $p=0.016$). Based on these results, group A pts were divided according to whether they had no, one or both, adverse prognostic factors identified in the multivariate analysis namely PS=2 and high *ERCC1* levels. Twenty pts (35%) had neither factor, 33 patients (58%) had one, and 4 (7%) had two. Median OS was 16.4 months (95% CI 12.4-18.6 months) for pts with neither factor, 12.1 months (95% CI 7.6-13.4 months) for pts with one factor, and 6.1 months (95% CI 3.5-10.7 months) for pts with two factors.

Gene expression levels and TTP. Median TTP in group A was longer for pts expressing low levels of *ERCC1*. The Cox regression analysis showed a correlation between TTP and both *ERCC1* ($p=0.087$) and PS 2 ($p=0.045$). In group B, no differences in TTP were observed with respect to *ERCC1* and all other clinical factors analyzed.

Gene expression levels and response to chemotherapy. The overall response rate to chemotherapy in this pooled patient population was 10%. Analyzing patients treated with platinum salts, there were significant differences in response according to *ERCC1* level. A low *ERCC1* level (28/57 pts) was highly predictive of a partial response (Figure 5).

Discussion

This retrospective study confirms prior data regarding second-line chemotherapy in pancreatic cancer, underlying that single-agent therapy for metastatic pancreatic cancer following gemcitabine is associated with a progression free survival (PFS) of less than 3 months. Fluoropyrimidine-based salvage regimens have marginal activity and should be considered only in pts with a good PS who have responded to first-line chemotherapy. This report indicates that low *ERCC1* expression correlates with increased OS in pts treated with cisplatin-based regimens as second-line therapy. For pts treated with fluoropyrimidine and platinum salts, a significantly longer median OS was observed in those with low *ERCC1* mRNA expression levels (11.9 months) compared to pts with higher levels (9.9 months), with a clear correlation between response to chemotherapy and low expression level. The univariate analysis confirmed our previous findings regarding the prognostic role of performance status, while other clinical characteristics failed to predict survival. When including *ERCC1* levels in both univariate and multivariate analysis, this marker also emerged as being independently associated with survival.

This report addresses the possible role of *ERCC1* expression in predicting OS of advanced pancreatic cancer patients treated with fluoropyrimidine and platinum chemotherapy as second-line therapy. In chemotherapy-naïve patients, five randomized trials have explored platinum combinations. They have included two oxaliplatin-based and three cisplatin-based combination studies. The platinum-based combinations induced a significant improvement of overall response rate and PFS in two trials (9-10), while the level of significance was not reached in three other trials (11-13). The platinum-based combination regimens consistently prolonged OS. None of the individual trials, however showed, statistically significant superiority compared to gemcitabine alone. A significant improvement of OS was detected only when a combined analysis of the five trials was performed (hazard ratio=0.85, $p=0.010$) (14).

In the second-line setting, there are no standard regimens for advanced pancreatic cancer. The CONKO-3 study randomized 168 patients who had gemcitabine-refractory pancreatic cancer to 5-FU, LV and oxaliplatin (OFF) or 5-FU and LV (FF). The study was powered at 90% to detect an improved OS by 2 months in the OFF arm. The median OS in the OFF arm was 28 weeks, and that of the FF arm was 13 weeks, thereby fulfilling the study hypothesis. There was also a significant prolongation of PFS in the investigational arm (13 *versus*. 9 weeks). To date, OFF can be considered as an option among the second-line regimens for pancreatic cancer patients (15).

The current report is consistent with second-line data in pancreatic cancer. This study has limitations, namely, that the genetic study, although carried out in a blinded fashion, was conducted retrospectively and there were several different platinum-containing regimens. In addition, this series might present a selection bias on the basis of better long-term outcome as a number of patients treated were treated with a

third-line of treatment with biological drugs. This point could explain the favorable results in terms of OS in the entire study population (11.5 months) and in the subgroup treated with platinum.

The strong association of *ERCC1* expression with survival still supports the hypothesis that enhanced DNA repair decreases the benefit of platinum-based treatment. Recent studies indicate that patients with lower DNA repair capacity are more chemosensitive than those who carry a proficient DNA repair system (16). DNA repair, especially nucleotide excision repair (NER), plays an important role in the defense of platinum-based drug-induced DNA damage, including the removal of DNA adducts (17). ERCC1 is the lead enzyme in the NER process. High ERCC1 levels are associated with increased removal of platinum-induced DNA adducts and relative platinum resistance (18), and both ERCC1-defective cells and knockout mice are highly sensitive to DNA cross-linking agents (19). Associations between *ERCC1* expression and survival outcome, with or without responsiveness to chemotherapy, have been previously documented in other platinum-sensitive tumor types (20-24). International results indicate that *ERCC1* may predict survival in bladder cancer treated by platinum-based therapy and finally, it has been recently shown that pts with ERCC1-negative non-small cell lung tumors appear to benefit from adjuvant cisplatin-based chemotherapy, whereas those with ERCC1-positive tumors do not (25-26).

There are limited data regarding molecular prognostic or molecular pharmacology predictive markers in pts with advanced pancreatic cancer. Ongoing trials and other prospective analyses will clarify the prognostic and predictive role of *ERCC1* and other factors (breast cancer gene 1 (*BRCA1*), ribonucleotide reductase subunit M1 (*RRM1*), caveolin-1) in this setting. At the moment, however, none of these molecular markers has yet proven useful in routine clinical practice. Optimizing chemotherapy with the use of chemosensitivity predictive markers such as intratumoral molecular pharmacology markers (pharmacogenomics) might help in improving outcome. Based on detailed molecular biological information of each tumor, the clinician may be able to more accurately select the appropriate therapy for each patient according to individual predicted response. In the current study, *ERCC1* is a novel marker of survival in pancreatic cancer. These preliminary results indicate that DNA repair genes may play an important role in the prognosis of advanced stage pancreatic cancer pts. Genetic testing of *ERCC1* mRNA expression levels could potentially be used to personalize chemotherapy by defining a subset of pts who would benefit the least from second-line platinum-based chemotherapy. These, with high *ERCC1* levels, would be the ideal target for novel therapeutic evaluation. Conversely, those with low levels may achieve a better outcome with second-line cisplatin-based chemotherapy. We believe that our data warrant further research. Further validation of these findings with larger sample sizes and prospectively randomized studies are needed to confirm these results and better define the clear biological basis of these findings.

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