Gynecologic Oncology 126 (2012) 448–454

Contents lists available at SciVerse ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Comparison of ERCC1/XPF genetic variation, mRNA and protein levels in women with advanced stage ovarian cancer treated with intraperitoneal platinum

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ARTICLE INFO

Article history:
Received 29 March 2012
Accepted 9 May 2012
Available online 16 May 2012

Keywords:
DNA repair
Platinum therapy
Chemoresistance
Biomarker
Prognosis
Single nucleotide polymorphism

ABSTRACT

Objective. Approximately 20% of patients receiving platinum-based chemotherapy for epithelial ovarian cancer (EOC) are refractory or develop early recurrence. Identifying these patients early could reduce treatment-associated morbidity and allow quicker transfer to more effective therapies. Much attention has focused on ERCC1 as a potential predictor of response to therapy because of its essential role in the repair of platinum-induced DNA damage. The purpose of this study was to accurately measure protein levels of ERCC1 and its essential binding partner XPF from patients with EOC treated with platinum-based therapy and determine if protein levels correlate with mRNA levels, patient genotypes or clinical outcomes.

Methods. ERCC1 and *XPF* mRNA and protein levels were measured in frozen EOC specimens from 41 patients receiving intraperitoneal platinum-based chemotherapy using reverse transcription polymerase chain reaction and western blots. Genotypes of common nucleotide polymorphisms were also analyzed. Patient outcomes included progression free (PFS) and overall survival (OS).

Results. Expression of *ERCC1* and *XPF* were tightly correlated with one another at both the mRNA and protein level. However, the mRNA and protein levels of ERCC1 were not positively correlated. Likewise, none of the SNPs analyzed correlated with ERCC1 or XPF protein levels. There was an inverse correlation between mRNA levels and patient outcomes.

Conclusion. Neither genotype nor mRNA levels are predictive of protein expression. Despite this, low *ERCC1* mRNA significantly correlated with improved PFS and OS.

Introduction

Epithelial ovarian cancer (EOC) remains the leading cause of death from gynecologic malignancy in this country. The majority of women present with advanced stage disease and are treated with aggressive surgery followed by platinum/taxane-based chemotherapy [1,2]. Seventy-five to 80% of women respond to this therapeutic regiment. The other 20–25% of patients are refractory, showing no response, or platinum resistance with cancer recurrence within 6 months of the end of treatment [3–5]. To date there is no way to predict who will respond to platinum-based chemotherapy and who is resistant and will suffer an early recurrence. Developing a strategy to identify patients who are resistant to standard first-line chemotherapy is a critical step toward improving treatment of patients with EOC [6,7].

The therapeutic effectiveness of platinum-based therapy is not fully understood, but its cytotoxicity is generally accepted to be mediated by the formation of platinum-DNA adducts [8]. Cisplatin forms primarily 1,2-intrastrand crosslinks between adjacent purines in DNA, and other adducts including 1,3 intrastrand crosslinks and interstrand crosslinks between the two strands of DNA [9]. The only mechanism for removing platinum intrastrand cross-links from DNA is nucleotide excision repair (NER) [10]. This mechanism requires over 30 proteins to recognize DNA adducts, excise them, and replace the missing nucleotides. The ERCC1-XPF heterodimer is an endonuclease essential for NER [11]. Approximately 5% of the DNA adducts formed by DNA cisplatin are interstrand crosslinks [9]. Interstrand crosslinks are extremely cytotoxic and thought to drive the anti-proliferative effects of crosslinking agents used in chemotherapy[12]. Interstrand crosslinks are repaired via a mechanism that is distinct from NER, but

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doi:10.1016/j.ygyno.2012.05.006

also requires the ERCC1-XPF nuclease [13]. Thus ERCC1 and XPF are the only two proteins that are absolutely required for the repair of all platinum-induced DNA adducts.

Based on this critical role in DNA repair, much effort has focused on whether *ERCC1* expression predicts tumor resistance to platinum therapy. In EOC, as with other tumor types, *ERCC1* genotype, mRNA levels, and protein levels have been speculated to reflect the functional level of ERCC1-XPF nuclease and thereby cellular DNA repair capacity [14–26]. In earlier studies, we demonstrated an association between ERCC1 genotype and clinical outcomes, which was especially pronounced in women treated through the intraperitoneal (IP) route [16]. Of note, the mechanism of ERCC1-XPF regulation has not yet been established and could be at the transcriptional, translational or post-translational level. Further, it is not known if ERCC1-XPF is rate limiting for NER or interstrand crosslink repair. While this does not exclude the utility of either the genotype or expression level of *ERCC1* as a clinically useful biomarker, the correlation may be unrelated to DNA repair.

It is important to address these gaps in knowledge by quantitatively measuring ERCC1-XPF mRNA and protein levels in a single set of tumor samples and determining if there is any correlation between these parameters and clinical outcomes. The aim of this study therefore was to utilize a well characterized tumor sample set to accurately measure ERCC1-XPF expression protein levels by immunoblot and determine if there is any correlation with mRNA levels, single nucleotide polymorphisms (SNPs) and clinical outcomes to gain mechanistic insight into the contribution of ERCC1-XPF to tumor resistance to platinum chemotherapy.

Methods

Patients and tissue

Forty-one de-identified frozen ovarian tumor samples were obtained from the Magee Womens Health Tissue Bank of the University of Pittsburgh Medical Center. Inclusion criteria consisted of women receiving IP platinum-based chemotherapy and a confirmed diagnosis of EOC. Patients were evaluated every 3 months for the first 2 years after surgery and then every 6 months for the next 3 years. Progression was defined by RECIST criteria or a doubling of CA125 from the laboratory normal. All samples and clinical data were collected through an honest broker system from patients who had given informed consent. This study was approved by the University of Pittsburgh Institutional Review Board.

Statistical analysis

Progression-free survival (PFS) and overall survival (OS) were measured from the date of surgery. PFS was the time until disease recurrence or death, whichever came first. OS was the time until death regardless of causes. Patients were grouped into three subgroups based on the level of *ERCC1* or *XPF* mRNA or protein expression (low, mid and high), with each subgroup including approximately the same number of patients. The Kaplan-Meier procedure was used to estimate the PFS and OS and the log-rank test was used to compare the group-difference in survival distributions. A Cox proportional hazards model was used to estimate the hazard ratio (HR) adjusted for age and stage. Associations between *ERCC1/XPF* genotype and mRNA/protein expression were evaluated using Wilcoxon rank-sum test. Spearman rank correlation was calculated to measure the relationship between *ERCC1* and *XPF* expression.

DNA isolation and genotyping

Genomic DNA was isolated from tissue using the Purgene Genomic DNA Purification Kit (Gentra, Minneapolis, MN) as described by the manufacturer's instructions. See supplemental data for genotyping details.

Measurement of ERCC1 and XPF mRNA

Total RNA was isolated from frozen tumor samples using Trizol reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Following isolation, DNA was removed by treatment with DNaseI (Invitrogen). RNA quality and quantity were determined by measuring absorption at 280 and 260 nm. cDNA was synthesized from 2 μg of total RNA using the High Capacity kit (Applied Biosystems; Foster City, CA). Expression of *ERCC1* (Assay ID # Hs00157415) and *XPF* (Hs00193342) was determined by real-time polymerase chain reaction (PCR) on an ABI Prism 7700 Sequence Detection System, according to the manufacturer's instructions (Applied Biosystems). PCR was performed using TaqMan® Gene Expression Assays (Applied Biosystems), which contain TaqMan unlabeled primers and a FAM (fluorescein) dye-labeled MGB (minor groove binder) probe for target genes. The *GAPDH* gene (Assay ID #Hs99999905) was used as an endogenous reference for all samples. All amplification cycles were performed in a single 50 μl reaction with cDNA equivalent to 100 ng of total RNA. A typical 50 μl reaction sample contained 25 μl of TaqMan Universal PCR Master Mix (containing 1X TaqMan buffer, 200 μM dNTPs, 5 mM $MgCl_2$, 1.25U AmpliTaqGold, and 0.5U of Amperase uracil-N-glycosylase (UNG) along with the TaqMan primers and MGB probes. Thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C followed by 45 cycles at 95 °C for 30 s and 60 °C for 1 min. Relative expression was determined using the comparative CT (Cycle-Threshold) method (Applied Biosystems, 2001), which consists of normalization of the number of target gene copies (*ERCC1* and *XPF*) to an endogenous reference gene (*GAPDH*). All experiments were conducted in duplicate and an average CT (Cycle-Threshold) value calculated for the replicates ± the standard error of the mean. The CT value refers to the cycle number wherein the fluorescent intensity crosses the threshold line, which is set in the exponential phase of the amplification plot above background levels.

Measurement of ERCC1 and XPF protein

Of the 41 patients, there was adequate frozen tumor specimen for quantitation of ERCC1 and XPF protein by immunoblot from 25 patients. Frozen samples were sonicated to homogenize the tissue in 1 mL of Laemmli's buffer (10% Glycerol, 50 mM Tris–HCl pH 6.8 and 2% SDS) containing 7 M urea, 5 mM dithiothreitol, 0.5 mM phenylmethyl sulfonyl fluoride, and 1 μg/ml each of protease inhibitors, leupeptin, aprotinin and pepstatin. The crude homogenate was cleared of debris by centrifugation at 10,000 rpm for 15 min at 4 °C. The supernatant was stored in aliquots at −80 °C until analyzed. 50 μg of total protein from each sample was resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was cut in half lengthwise. XPF was detected in the upper portion of the membrane (MW 120 kDa) with primary antibody Ab-1 (mouse monoclonal, Neomarkers, 1:1000) and alkaline phosphatase (AP)-conjugated goat anti-mouse secondary antibody (1:7500, Promega). ERCC1 was detected in the lower portion of the membrane (MW 37 kDa) with primary antibody FL-297 (rabbit polyclonal, Santa Cruz, 1:1000) and AP-conjugated goat anti-rabbit secondary antibody (1:7500, Promega). The loading control, β-actin, was also detected in the lower portion of the membrane (MW 45 kDa) with primary antibody ab13822 (chicken monoclonal, 1:1000, Abcam,) and AP-conjugated goat anti-chicken secondary antibody (1:1000; Abcam). Recombinant ERCC1-XPF [46] was used as an internal standard for the electrophoretic mobility of ERCC1 and XPF. Protein levels were quantitated by densitometry and corrected for loading using the actin control.

Results

Tumor specimens

Frozen samples of tumors were obtained from 41 patients presenting to Magee Womens Hospital with EOC and who consented to