Keap1 Mutations and Nrf2 Pathway Activation in Epithelial Ovarian Cancer

Panagiotis A. Konstantinopoulos¹, Dimitrios Spentzos¹, Elena Fountzilas¹, Nancy Francoeur¹, Srisowmya Sanisetty¹, Alexandros P. Grammatikos², Jonathan L. Hecht³, and Stephen A. Cannistra¹

Abstract

Resistance to platinum-based chemotherapy develops in the majority of patients with epithelial ovarian cancer (EOC). Platinum compounds form electrophilic intermediates that mediate DNA cross-linking and induce double-strand DNA breaks. Because the cellular response to electrophilic xenobiotics is partly mediated by Keap1–Nrf2 pathway, we evaluated the presence of Kelch-like ECH–associated protein 1 (Keap1) mutations and NF-E2–related factor 2 (Nrf2) pathway activation in EOC and correlated these with platinum resistance and clinical outcome. Nrf2 immunohistochemistry revealed nuclear localization (a surrogate of pathway activation) in over half of EOC patient specimens examined, with more common occurrence in the clear cell EOC subtype. Quantitative real-time PCR revealed that Nrf2 target genes were upregulated in tumors with nuclear positivity for Nrf2. Microarray analysis also showed upregulation of Nrf2 target genes in clear cell EOCs compared with other EOC subtypes. In addition, Keap1 sequence analysis revealed genetic mutations in 29% of clear cell samples and 8% of nonclear cell tumors. RNAi-mediated knockdown of Keap1 was associated with Nrf2 pathway activation and resistance to carboplatin in vitro. Importantly, patients with evidence of Nrf2 pathway activation had fewer complete clinical responses to platinum-based therapy, were enriched for platinum resistance, and had shorter median overall survival compared with those who did not show evidence of Nrf2 pathway activation. Our findings identify Keap1 mutations in EOC and they suggest a previously unrecognized role for the Keap1–Nrf2 pathway in mediating chemotherapeutic responses in this disease. Cancer Res; 71(15); 5081–9. ©2011 AACR.

Introduction

Resistance to active drugs such as platinum compounds commonly occurs during treatment of epithelial ovarian cancer (EOC; refs. 1, 2). These drugs form electrophilic intermediates that act via nucleophilic substitution reactions to form inter- and intrastrand DNA crosslinks. The cellular response to electrophilic xenobiotics is partly mediated by the Keap1–Nrf2 signaling system (3). NF-E2–related factor 2 (Nrf2) is a leucine zipper transcription factor, which under basal conditions is tethered to its cytoplasmic inhibitor Kelch-like ECH–associated protein 1 (Keap1). Upon binding to Nrf2, Keap1 inhibits Nrf2 function by promoting degradation of Nrf2 (4). Electrophilic xenobiotics trigger the disassociation of Nrf2 from Keap1, allowing subsequent translocation of Nrf2 into the nucleus. Nrf2 subsequently activates transcription of a variety of genes whose protein products protect against oxidative stress, mediate detoxification of substances through glutathione conjugation, and participate in ATP-dependent drug efflux (5, 6).

The role that the Keap1–Nrf2 pathway plays in the development of drug resistance in EOC is poorly understood. In this study, we assessed the prevalence of Keap1 mutations and Nrf2 pathway activation in EOC, and the relationship between Nrf2 pathway activation, platinum resistance, and clinical outcome.

Materials and Methods

Patient samples and cell culture

Formalin-fixed, paraffin-embedded tissue from 30 EOC patients treated at Beth Israel Deaconess Medical Center (BIDMC) were included in this study (Supplementary Table 1). The study protocol for collection of tissue and clinical information was approved by the Institutional Review Board at BIDMC. The 36M2 human EOC cell line was derived from serial passage of ovarian serous carcinoma cells in nude mice as previously described (7).
Immunohistochemistry

Immunohistochemistry (IHC) to detect nuclear expression of Nrf2 was determined as described in Supplementary Methods. Cases displaying nuclear Nrf2 immunostaining in more than 10% of tumor cells were regarded as positive.

Real-time PCR

Total RNA was isolated from 36M2 cells using RNeasy Mini Kit (Qiagen). Reverse transcription was done with 1 μg starting total RNA and the Promega Reverse Transcription System (Promega) according to the manufacturer’s instructions. SYBR Green I-based real-time PCR was carried out on a MJ Research DNA Engine Opticon Continuous Fluorescence Detection System.

Total RNA was isolated from formalin-fixed, paraffin-embedded tissue sections using RNeasy FFPE Kit (Qiagen). RNA was reverse transcribed into complementary DNA (cDNA) with Promega’s Reverse Transcription System according to the manufacturer’s instructions. Real-time PCR was set up with Roche Universal Probe Library hydrolysis probes and Probes Master reagents and amplification was done in triplicate on the LightCycler 480 (Roche). The primers used are listed in supplement methods and were designed using ProbeFinder online tool from Roche. Relative quantitative levels of samples were determined by the 2-ΔΔCt method and for genes with decreased expression the negative inverse value was used.

Transfection and siRNA knockdown of Keap1

Nontargeting scramble and Keap1-targeting siRNA sequences were obtained from Dharmacon. Transfections were done with cells at 30% to 40% confluence using Lipofectamine 2000 (Invitrogen) and OPTI-MEM-reduced serum medium (Invitrogen) as per the manufacturer’s instructions. Final Keap1-targeting siRNA and concentration were chosen on the basis of dose–response studies of Keap1 knockdown. All experiments were done 3 times.

Keap1 and Nrf2 sequence analysis

DNA was isolated from formalin-fixed cancer tissue specimens using QiAamp DNA FFPE Tissue Kit (Qiagen). All protein-coding exons and intron–exon boundaries of the Keap1

<table>
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<th>Characteristic</th>
<th>NRF2 positive* ( (n = 17) )</th>
<th>NRF2 negative ( (n = 13) )</th>
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<th>( p^b )</th>
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<td></td>
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<td>2</td>
<td>3</td>
<td></td>
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<tr>
<td>Suboptimal</td>
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<td>4</td>
<td>7</td>
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</tr>
</tbody>
</table>

*Samples with greater than or equal to 10% of cells with nuclear stain positivity for Nrf2 were considered positive.

bTwo-sided Fisher’s exact test except otherwise specified.

c\( t \) test.

dStage was unknown for 1 patient.

eOptimal debukling was defined as less than or equal to 1 cm of gross residual disease, and suboptimal debukling was defined as greater than 1 cm of residual disease.

fDebulking status was unknown for 1 patient.

gClear cell versus all remaining histologies.

hEarly stage (1, 2) versus advanced stage (3, 4).
and Nrf2 gene were amplified and sequenced using a 2 step "boost/nest" PCR strategy. Bidirectional DNA sequencing was conducted, and the sequences of all the primers (boost and nest primers) are provided in Supplementary Table S2 for both Keap1 and Nrf2. All reported sequence alterations were confirmed by sequencing in both directions and the associated base call accuracy was more than 99% in all cases.

Microarray datasets and analysis

Two publicly available microarray datasets were used in this study and have been previously described by our group and others (8, 9). Hierarchical clustering based on the expression of Nrf2 pathway genes (i.e., genes of the Biocarta Nrf2 pathway including the experimentally validated Nrf2 target genes SOD2, GPX3, SLC3A1, GGT1, GSTM3, CRYZ, POR, and NQO1) was conducted by using the average linkage method. Pathway analysis was done by using the Efron Tibshirani’s method, and statistical significance was assessed by using the log-rank test. Multivariate analysis was done by using a Cox proportional hazards regression model that included grade (1–2 vs. 3), age (<65 vs. ≥65 years), stage (2 vs. 3 or 4), histology (clear cell, papillary serous, endometrioid), debulking status (optimal, less than or equal to 1 cm; or suboptimal, greater than 1 cm residual disease), and Nrf2 pathway status (Nrf2 pathway up vs. Nrf2 pathway down). For the purpose of this report, clinical complete remission (CCR) is defined as resolution of all clinical and radiographic evidence of disease and normalization of the serum CA-125 level after the completion of first-line chemotherapy, whereas platinum resistance is defined as progressive disease on platinum therapy, or less than a complete response to platinum therapy, or progression within 6 months of completing platinum therapy.

Results

Nrf2 pathway activation in EOC

We conducted IHC to assess the expression and localization of Nrf2 protein in 30 EOC specimens obtained during primary debulking surgery, with the results shown in Table 1. Nuclear Nrf2 staining was detected in 17 (57%) of EOC specimens overall and was more common in clear cell EOCs (11 of 14, 79%) compared with nonclear cell EOC (6 of 16, 38%), 2-sided Fisher’s exact P = 0.033; Table 1, Fig. 1A–C). There were no differences in age, grade, or debulking status between Nrf2 immunopositive and negative specimens (Table 1). Two normal Mullerian epithelium specimens showed no evidence for Nrf2 nuclear protein by IHC (Fig. 1B).

To evaluate whether presence of nuclear Nrf2 staining was associated with Nrf2 transcriptional activity, we compared the expression levels of 2 Nrf2 target genes (SOD2 and GPX3) in Nrf2 immunopositive and immunonegative specimens using quantitative real-time PCR. We found that both genes were statistically significantly upregulated in Nrf2 immunopositive compared with immunonegative specimens (16.3- and 13.8-fold for SOD2 and GPX3 respectively, P < 0.05 for both genes, Fig. 2A). Furthermore, we found that both SOD2 and GPX3 were statistically significantly upregulated in clear cell carcinomas compared with nonclear cell tumors (6- and 23.3-fold for SOD2 and GPX3 respectively, P < 0.05 for both genes).

We next evaluated the expression of more Nrf2 target genes in clear cell and nonclear cell EOCs using gene expression data from a publicly available, clinically annotated ovarian cancer microarray dataset (8). Nrf2 target genes were statistically significantly overexpressed in clear cell EOCs compared with the other major EOCs histologic types (Fig. 2B). The fact that expression of known Nrf2 target genes (i.e., genes of the Biocarta Nrf2 pathway and NQO1).

Statistical analysis

The association between Nrf2 IHC results and various clinicopathologic factors was assessed by Fisher’s exact test. Overall survival (OS) curves were generated by the Kaplan–Meier method, and statistical significance was assessed by using the log-rank test. Multivariate analysis was done by using a Cox proportional hazards regression model that included grade (1–2 vs. 3), age (<65 vs. ≥65 years), stage (2 vs. 3 or 4), histology (clear cell, papillary serous, endometrioid), debulking status (optimal, less than or equal to 1 cm; or suboptimal, greater than 1 cm residual disease), and Nrf2 pathway status (Nrf2 pathway up vs. Nrf2 pathway down). For the purpose of this report, clinical complete remission (CCR) is defined as resolution of all clinical and radiographic evidence of disease and normalization of the serum CA-125 level after the completion of first-line chemotherapy, whereas platinum resistance is defined as progressive disease on platinum therapy, or less than a complete response to platinum therapy, or progression within 6 months of completing platinum therapy.

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genes was found to be upregulated more commonly in clear cell EOCs compared with nonclear cell histologies provides preliminary evidence to suggest that the nuclear location of Nrf2 in this relatively chemoresistant ovarian cancer subtype may have functional significance. Also, pathway analysis revealed that Nrf2 pathway was upregulated in clear cell EOCs (Efron–Tibshirani’s GSA test, \( P < 0.005 \)) compared with nonclear cell EOCs. Furthermore, as shown in the hierarchical clustering of

Figure 2. Upregulation of Nrf2 target genes in Nrf2 positive tumors and clear cell EOCs. A. fold upregulation of Nrf2 target genes in tumors with positive nuclear staining for Nrf2 (\( n = 17 \)) compared with tumors with no nuclear staining for Nrf2 (\( n = 13 \)) as assessed by quantitative real-time PCR. GPX3, glutathione peroxidase-3; SOD2, superoxide dismutase-2. Asterisks indicate statistically significant results (\( P < 0.05 \)). B, fold upregulation of Nrf2 target genes in clear cell samples compared with nonclear cell samples assessed by microarray analysis. Asterisks indicate statistically significant results (\( P < 0.05 \)). SLC3A1, solute-carrier family-3A1; GGT1, gamma glutamyltransferase-1; GSTM3, glutathione-S-transferase-M3; CRYZ, quinone reductase; POR, P450-oxidoreductase. Asterisks indicate statistically significant results (\( P < 0.05 \)). C, hierarchical clustering based on the expression pattern of Nrf2 pathway genes reveals that all clear cell EOCs cluster together, that is, have a distinct genomic profile.

Figure 3. Keap1 mutations in EOC. A, location of mutations within Keap1 protein. NTD, N-terminal domain; BTB, broad complex-Tramtrack-Bric-a-brac; IVR, Intervening region; KR, Kelch repeat; CTD, C-terminal domain. \( ^{a} \) Numbers correspond to Figure 2B, \( ^{b} \) patient numbers correspond to Supplementary Table S1. B, sequence analysis of Keap1 mutations in EOC. Electropherograms depicting Keap1 mutations identified in EOC. T, Keap1 sequence of EOC sample harboring the mutation; N, normal Keap1 sequence.
tumors based on the Nrf2 pathway genes (Fig. 2C), all clear cell tumors cluster together, suggesting that clear cell tumors have a distinct genomic pattern of Nrf2 gene expression when compared with nonclear cell tumors. The results of pathway analysis and hierarchical clustering did not change if additional Nrf2 pathway genes were included in the analysis [i.e., those present in the publicly available signature ("SINGH-NFE2L2-TARGETS").

Keap1 mutations in EOC

Heterozygous mutations in Keap1, associated with subsequent activation of the Nrf2 pathway, have been identified in lung and biliary tract cancer (11–13). We therefore amplified and sequenced all 5 protein-coding exons and intron–exon boundaries of the Keap1 gene in 27 of the 30 EOC samples, with the results shown in Figures 3A and B and Supplementary Table S3. We identified mutations associated with changes in evolutionarily conserved amino acids (in Macaca mulatta, Mus musculus, Danio rerio (zebrafish), Xenopus laevis, and Drosophila) in 4 of 14 (29%) clear cell samples. Three clear cell tumors were heterozygous for the mutant allele whereas the fourth was heterozygous for 2 different mutations. Keap1 expression levels and Nrf2 gene sequencing in Nrf2 immunopositive specimens

In addition to Keap1 mutations, we considered that Nrf2 pathway activation may also occur as a result of Keap1 downregulation or Nrf2 mutations. Therefore, we analyzed the mRNA expression levels of Keap1 by quantitative reverse transcriptase PCR (qRT-PCR) and conducted sequencing of Nrf2 gene to identify mutations in Nrf2 immunopositive tumors. Table 2 summarizes the Keap1 expression data, and the Keap1 and Nrf2 sequencing data in the Nrf2 immunopositive tumors.

As shown in Table 2, 3 tumors (all with clear cell histology) with positive Nrf2 nuclear staining had absent Keap1 expression (i.e., Keap1 transcript was undetected by RT-PCR after 55 cycles of amplification (reactions conducted in triplicate)).

Keap1/Nrf2 Pathway in Epithelial Ovarian Cancer

Table 2. Keap1 and Nrf2 sequence alterations, and Keap1 expression in Nrf2 immunopositive patients

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<th>Patient no.</th>
<th>Nrf2 IHC</th>
<th>Histology</th>
<th>Keap1 expression*</th>
<th>Keap1 sequence alterations</th>
<th>Nrf2 sequence alterationsb</th>
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</tr>
<tr>
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<td>8</td>
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<td>None</td>
</tr>
<tr>
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<tr>
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<tr>
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*aKeap1 expression was determined by RT-PCR as described in text. *Present* refers that Keap1 transcript was detected after 55 cycles of amplification. *Absent* refers to the fact that no Keap1 transcript was detected by RT-PCR after 55 cycles of amplification (reactions conducted in triplicate).

bBidirectional DNA sequencing of Nrf2 was conducted, and the sequences of all the primers are provided in Supplementary Table S2.
Overall, of 17 tumors with positive nuclear Nrf2 staining, 8 (47%) tumors had either Keap1 mutations (n = 5) or absent Keap1 mRNA expression (n = 3). Of 11 clear cell tumors with positive nuclear Nrf2 staining, 7 (64%) tumors had either Keap1 mutations (n = 4) or absent Keap1 mRNA expression (n = 3).

Activation of the Nrf2 pathway via siRNA knockdown of Keap1

We assessed the effect of siRNA knockdown of Keap1, the cytoplasmic inhibitor of Nrf2, on the sensitivity of 36M2 ovarian cancer cells to carboplatin. siRNA against Keap1 was transfected into 36M2 cells (which harbor wild-type Keap1) at a dose of 150 nmol/L. After 48 and 72 hours, siRNA against Keap1 reduced the steady-state mRNA levels of Keap1 (assessed by real-time RT-PCR) to 40% of that observed in untreated 36M2 cells or those transfected with the scrambled siRNA (Fig. 4A). siRNA knockdown of Keap1 was maintained at this level for 72 hours (Fig. 4A). To confirm that knockdown of Keap1 allowed Nrf2 to activate transcription of its target genes, we assessed expression of the Nrf2 target gene NQO1, quinone oxidoreductase 1 (NQO1) after siRNA knockdown of Keap1. siRNA against Keap1 produced a 2.5-fold and 10-fold increase in NQO1 mRNA levels at 48 hours and 72 hours, respectively, compared with cells treated with scrambled siRNA or control, and remained upregulated at 96 hours after transfection (Supplementary Fig. S1).

As shown in Figure 4B, transfection with Keap1 siRNA induced relative resistance of 36M2 cells to carboplatin, compared with 36M2 cells transfected with scrambled siRNA or control 36M2 cells. For cells transfected with Keap1 siRNA, scramble siRNA, and control, the IC50 to carboplatin was 49.4, 11.5, and 12.1 µmol/L, respectively (P < 0.05 for the difference between Keap1 siRNA and either control group).

Correlation of Keap1–Nrf2 pathway with platinum responsiveness and clinical outcome

We used our publicly available ovarian cancer microarray dataset comprised of 64 advanced disease patients with EOC to investigate whether there was a relationship between Nrf2 pathway activation, platinum resistance, and OS (9). Hierarchical clustering based on the expression levels of the Nrf2 pathway genes revealed 2 clusters/groups of patients (Fig. 5A). The Nrf2 pathway was upregulated (Efron–Tibshirani’s GSA test P < 0.005) in the first group (Nrf2 pathway up) compared with the second group (Nrf2 pathway down) of patients (heat map is shown in Supplementary Fig. S2). The majority of clear cell EOCs (4 of 5, 80%) clustered in the "Nrf2 pathway up" group and 1 clustered in the "Nrf2 pathway down" group.

Patients in the Nrf2 pathway up group had a lower complete clinical response rate to platinum, compared with patients in the Nrf2 pathway down group (77% vs. 97%, respectively, 2-sided Fisher’s exact P = 0.015; Fig. 5B). Furthermore, 42% of patients in the Nrf2 pathway up group were platinum resistant, compared with 16% of patients in the Nrf2 pathway down group (2-sided Fisher’s exact P = 0.02; Fig. 5B). Finally, patients in the Nrf2 pathway up group had inferior OS compared with patients in the Nrf2 pathway down group (34 months vs. not yet reached, respectively, log rank P = 0.031, Fig. 5C). In multivariable analysis adjusted for known prognostic factors, the HR for death for patients in the Nrf2 pathway up group was 2.42 (95% CI: 1.15–5.09; Supplementary Table S4).

Discussion

Platinum compounds form electrophilic intermediates that mediate DNA cross-linking and induce double-strand DNA breaks. Because the cellular response to electrophilic xenobiotics is partly mediated by Keap1–Nrf2 pathway, we were interested in further defining the role that this pathway might play in the development of drug resistance in EOC. We report for the first time that Nrf2 is commonly localized to the
nucleus in EOC (Fig. 1), associated with evidence of downstream activation of Nrf2 target genes, resistance to platinum, and mutations in its binding partner, Keap1. This effect was most commonly observed for clear cell EOC, a subtype that is already known to have a distinct molecular profile compared with other ovarian cancer histologies (17). Although nuclear localization of Nrf2 by IHC has been reported in non–small cell lung and head and neck cancers, this is the first report to show that this phenomenon also occurs in EOC (13, 18). The fact that expression of known Nrf2 target genes was found to be upregulated more commonly in clear cell EOCs compared with nonclear cell histologies (Fig. 2), provides supportive evidence to suggest that the nuclear location of Nrf2 in this ovarian cancer subtype may have functional significance.

To assess whether activation of the Nrf2 pathway may be partly responsible for mediating platinum resistance in EOC, we evaluated the functional relationship between Nrf2 pathway activation and platinum resistance in the 36M2 ovarian cancer cell line. Nrf2 pathway was induced via siRNA knockdown of its cytoplasmic inhibitor Keap1 (Fig. 4A), leading to enhanced the transcriptional activity of Nrf2 and rendering 36M2 cells more resistant to carboplatin (Fig. 4B).

Keap1 mutations have been reported in some patients with non–small cell lung cancer (13, 11), small cell lung cancer (11), and biliary tract cancer (12). One study also reported that the human Q293 breast cancer cell line harbors a Keap1 mutation that encodes an inactive Keap1 protein (19). The majority of these Keap1 mutations in cancer patients were heterozygous. In our study, we identified mutations associated with changes in amino acids in 5 (19%) of the 27 EOCs that were sequenced; in 4 (29%) clear cell samples and 1 (8%) nonclear cell (papillary serous) cancer (Table 2). All EOC tumors with Keap1 mutations exhibited nuclear localization of Nrf2 by IHC in our series. Three clear cell tumors were heterozygous for the mutant allele whereas the fourth was heterozygous for 2 different mutations. Importantly, all 5 missense mutations identified in the 4 clear cell tumors involved amino acids that were evolutionary conserved in Macaca mulatta, Mus musculus, Danio rerio (zebrafish), Xenopus laevis, and Drosophila. Similar to other tumor types, clear cell EOC samples were heterozygous for Keap1 mutations, and these mutations involved functionally relevant domains of Keap1 protein, including the BTB, IVR, and KR regions of Keap1 which are responsible for ubiquitination and binding of Nrf2 (14–16). On the basis of the recently proposed 2-site substrate recognition model for Keap1–Nrf2 interaction, formation of an homodimer of 2 normal Keap1 subunits that associates with 2 sites of Nrf2 is necessary for ubiquitination and eventual degradation of Nrf2 (20). In this regard, it has been reported that in tumors that are heterozygous for Keap1 mutations, the heterodimer between a wild-type Keap1 subunit and a mutant Keap1 subunit is inactive and unable to repress Nrf2 function (11). Finally, there was 1 missense mutation in 1 papillary serous tumor, which contained only the mutant G–C allele, associated with a highly conserved amino acid change in the BTB domain (Fig. 3 and Supplementary Table S3).
We also evaluated whether other mechanisms (besides Keap1 mutations) may be associated with Nrf2 pathway activation in EOC. In this regard, downregulation of Keap1 and mutations in Nrf2 have been identified in lung cancer (21). Therefore, we analyzed the expression levels of KEAP1 by qRT-PCR and conducted sequencing of NFE2 gene to identify mutations in the Nrf2 immunopositive tumors. No NRF2 sequence alterations were identified in the Nrf2 immunopositive tumors (Table 2). However, we found that there were 3 tumors (all clear cell tumors) with positive Nrf2 nuclear staining that had absent Keap1 expression as assessed by qRT-PCR (Table 2). None of these tumors harbored Keap1 mutations. Thus, of 17 tumors with positive nuclear Nrf2 staining, 8 (47%) tumors had either Keap1 mutations or absent Keap1 mRNA expression, and of 11 clear cell tumors with positive nuclear Nrf2 staining, 7 (64%) tumors had either Keap1 mutations or absent Keap1 mRNA expression.

Given the presence of Nrf2 pathway activation in at least some patients with EOC, and the observation that activation of Nrf2 pathway increases platinum resistance in 36M2 ovarian cancer cells, we investigated whether this pathway is associated with clinical outcome in ovarian cancer using a publicly available gene expression dataset of 64 patients with advanced EOC. Patients with upregulated Nrf2 pathway had a lower CCR rate after first-line therapy, and they were enriched for platinum resistance. Furthermore, activation of Nrf2 pathway was associated with worse OS independent of standard prognostic factors such as age, grade, histology, stage, and debulking status (Supplementary Table S4). Because the majority of the 64 patients had papillary serous tumors, these findings suggest that activation of the Nrf2 pathway may correlate with platinum resistance not only in clear cell EOC but also in other histologies.

In conclusion, the Nrf2 pathway is activated at baseline in some patients with EOC, especially of the clear cell variety, and may be partly involved in the development of chemoresistance of this disease. Activation of Nrf2 pathway in EOC seems to be associated with Keap1 mutations within highly conserved domains of the Keap1 gene, raising the possibility that such mutations may prove to be a surrogate for platinum resistance. Alternatively, activation of Nrf2 pathway may be associated with downregulation of Keap1 expression. If confirmed in a larger numbers of patients, these findings also suggest that Nrf2 may serve as an important therapeutic target for novel drugs capable of preventing or reversing resistance to chemotherapy in EOC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Panagiotis A. Konstantinopoulos, Dimitrios Spentzos, Elena Fountzilas, et al.

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