Correlations among ERCC1, XPB, UBE2I, EGF, TAL2 and ILF3 revealed by gene signatures of histological subtypes of patients with epithelial ovarian cancer

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Received August 17, 2011; Accepted September 12, 2011

DOI: 10.3892/or.2011.1483

Abstract. The aim of this study was to better understand the mechanisms of tumor development and disease progression in human epithelial ovarian cancer. Fifty genes were screened for gene signature; 20 expressed genes were assessed in tumor and normal samples of EOC patients by RT-PCR. Expression of UBE2I, EGF, TAL2 and ILF3 was validated by qPCR on the ABI PRISM 7000 Detection System. ERCC1 and XPB expression was previously determined by RT-PCR in these specimens. Statistical analyses include two-sided Kruskal-Wallis test, pairwise comparison, Pearson correlation coefficient and paired t-test. In comparison to normal samples, 6 genes demonstrated distinct expression patterns in tumor tissues, with high expression observed for ERCC1, XPB and ILF3 ($p=0.001$, 0.0007 and 0.002, respectively) and low expression observed for TAL2 and EGF (both $p<0.0001$). This differential expression pattern between normal and tumor tissues may reflect in part the development of ovarian cancer. Significant differences in expression patterns of these genes in clear cell, endometrioid, mucinous and serous ovarian cancer were observed. Comparison of expression of any two EOC subtypes revealed multiple gene involvement in histopathological differentiation and cancer progression. A positive association was found between ERCC1 and XPB expression ($r=0.53$, $p<0.0001$) and between TAL2 and EGF expression ($r=0.817$, $p<0.0001$) suggesting the existence of gene linkage in these tumors. The differences in expression patterns of studied genes between tumors and normal specimens, between histological subtypes and correlations among studied genes, may indicate their involvement in tumor growth and disease progression in human epithelial ovarian cancer. Further investigation of these genes may enable better understanding of the molecular mechanism of tumorigenesis and identification of potential biomarkers.

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Key words: gene signature, epithelial ovarian cancer, histological subtypes, expression pattern

Introduction

Human epithelial ovarian cancer (EOC), comprising the majority of malignant ovarian tumors in adult women, is the most lethal form of gynecologic cancer and the fourth leading cause of death among women in developed countries. In the United States around 21,880 new cases and 13,850 deaths occurred in 2010 (1). However, if diagnosis is made at stage I, with malignancy confined to the ovary, the survival rate can reach approximately 94%. Otherwise, the 5-year survival rates sharply decline to 28% at advanced stages (1,2). Inadequate biomarker(s) and lack of an effective screening strategy for early detection result in more than 80% of patients presenting with advanced disease. The test of serum marker CA-125 (cancer antigen 125) has been clinically used for ovarian cancer, but it lacks sensitivity or specificity to function alone as an early screening test. Combining CA-125 and ultrasound or computed tomographic scan is an improvement, but still not sensitive enough for early detection of this disease (2-4).

On the other hand, drug resistance in cancer treatment is a major contribution to the very high mortality rates. In advanced ovarian cancer, the initial response of first-line chemotherapy with platinum compounds is more than 80%, with 40-60% complete response (5), but median progression-free survival is only 18 months in these patients, as most relapse. The overall response rate in platinum-refractory or drug-resistant tumors is only 10-25% due to relapse, resulting in a 5-year survival of only 25-30% (1,5).

Table I. The Difference in gene expression between normal and tumor tissues examined by paired t-test.

	n	Mean	Median (range)	SEM	p-value
ERCC1					
Tumor tissues	47	1.15	0.76 (0, 6.02)	0.20	0.001
Normal tissues	47	0.50	0.09 (0, 4.09)	0.12	
XPB					
Tumor tissues	47	0.95	0.91 (0, 2.64)	0.11	0.0007
Normal tissues	47	0.45	0.04 (0, 2.18)	0.10	
ILF3					
Tumor tissues	41	595.64	187.01 (0.34, 4389.98)	165.21	0.002
Normal tissues	41	46.80	2.03 (0.03, 498.69)	18.21	
TAL2					
Tumor tissues	47	3.38	0.96 (0.02, 63.56)	1.38	<0.0001
Normal tissues	47	14.48	10.26 (0.19, 58.16)	1.99	
EGF					
Tumor tissues	41	1.92	0.99 (0.03, 14.53)	0.45	<0.0001
Normal tissues	41	11.26	9.27 (0.11, 35.14)	1.60	
UBE2I					
Tumor tissues	47	76.14	3.59 (0.18, 1956.42)	41.71	0.06
Normal tissues	47	1136.58	1.56 (0.05, 14623.49)	544.25	

In addition, clinicians have long known that histological types of ovarian cancer respond differently to treatment and have different prognoses (6,7). Our previous studies indicate that different subtypes of epithelial ovarian cancer show different levels of *de novo* drug resistance against DNA-damage agents in the clinic (6); genes involved in transcriptional regulation, DNA repair, and apoptosis show high expression levels in advanced stage and advanced grade ovarian cancer patients (8). Others have reported that molecular genetic alterations and differential gene-expression are associated with histological subtypes and contribute to carcinogenesis and progression of this disease (9-13). Approximately 10% of ovarian cancers are familial and relate to certain gene mutations. High-grade serous ovarian cancers harbor mutations of p53, BRCA1 or BRCA2, and the extremely low BRCA1 constitutive-expression plays a role in tumorigenesis of the ovary. For EOC, the morphologically and biologically heterogeneous disease, each subtype represents distinct disease entities and is more likely affected by multiple gene-activation and -alteration in the etiologic mechanism (14-17). Therefore, better understandings of the mechanisms of tumor development and refractory tumors are critical in successfully fighting this disease.

Materials and methods

Human samples and ethics statement. Frozen GOG samples from patients with advanced ovarian cancer were obtained from Cooperative Human Tissue Network (CHTN), Pediatric Division, Children's Hospital, Columbus, OH. Samples were stripped of all patient identifiers before shipping. There is no way to link molecular laboratory data with any subject in this study. The 83 tumor specimens and 48 adjacent normal ovarian tissues were collected at primary surgery prior to chemotherapy, flash frozen in liquid nitrogen and stored at -80˚C until RNA/DNA extraction. All samples were evaluated by pathologists and stratified as serous carcinoma (n=51), endometrioid carcinoma (n=13), mucinous carcinoma (n=11) or clear cell carcinoma (n=8).

RNA extraction. Total RNA from each of the 83 tumor and 48 normal specimens of ovarian cancer patients was extracted and purified by the method of hot phenol/chloroform extraction as previously reported (6). Isolated RNA was precipitated and dissolved in DEPC water and stored at -80˚C.

Oligo synthesis. Fifty genes, with the exception of ERCC1 and XPB, which were previously studied in our lab, were selected by searching gene databases, based on gene function and published significant expression-patterns in ovarian, breast and lung cancers. Primary gene functions for this investigation included transcription factor, gene activation and regulation, and binding activities. Forward and reverse primers for each of the 50 genes were designed and synthesized commercially (8).

Reverse transcription PCR. The selected 50 genes (exclusive of ERCC1 and XPB) were screened by RT-PCR in a separate panel of 28 ovarian cancer tissues. Twenty expressed genes of the initial 50 genes were further assessed in a panel of 48 paired tumor and normal plus 35 tumor specimens of stage III/IV advanced ovarian cancer patients. In brief, cDNA was generated using 5 μg of total RNA from tumor and normal samples. Reverse-transcription PCR (RT-PCR) was performed with cDNAs, AmpliTaq DNA polymerase and gene-specific primers at optimal PCR conditions. β-actin served as endogenous control. PCR amplicons were separated by gel electrophoresis and visible density was achieved by Southern blotting with

Table II. The difference in gene expression among histological subtypes examined by Kruskal-Wallis test.

Tumor type	n	Mean	Median (range)	SEM	p-value
ERCC1					
Clear cell	9	1.47	1.21 (0.17, 3.09)	0.35	
Endometrioid	21	0.57	0.49 (0, 2.07)	0.13	0.004
Mucinous	11	0.31	0.05 (0, 1.15)	0.13	
Serous	76	0.92	0.75 (0, 6.02)	0.14	
XPB					
Clear cell	9	1.25	1.18 (0.41, 2.64)	0.24	
Endometrioid	21	0.77	0.68 (0, 4.28)	0.22	0.015
Mucinous	11	0.38	0 (0, 2.14)	0.22	
Serous	76	0.86	0.84 (0, 3.41)	0.08	
TAL2					
Clear cell	8	0.71	0.28 (0.01, 3.05)	0.37	
Endometrioid	13	2.98	1.57 (0.01, 9.15)	0.95	0.003
Mucinous	11	4.97	3.65 (0.54, 14.2)	1.29	
Serous	49	2.45	0.73 (0, 63.56)	1.29	
EGF					
Clear cell	6	1.31	0.98 (0.02, 4.14)	0.62	
Endometrioid	11	3.72	2.81 (0.04, 14.53)	1.32	0.028
Mucinous	11	2.87	2.4 (0.63, 6.54)	0.62	
Serous	41	1.23	0.66 (0.03, 9.18)	0.29	
UBE2I					
Clear cell	8	29.17	10.14 (0.88, 97.68)	12.84	
Endometrioid	13	14.89	1.7 (0.43, 86.94)	7.13	0.013
Mucinous	10	3.68	1.09 (0.53, 14.92)	1.66	
Serous	49	614.10	22.35 (0.18, 13088.39)	367.81	
ILF3					
Clear cell	8	571.10	588 (3.93, 1677.39)	196.19	
Endometrioid	12	289.75	36.92 (1, 1773.03)	157.57	0.061
Mucinous	6	68.82	5.69 (1, 308.9)	50.06	
Serous	48	870.15	328.11 (0.34, 16270.83)	350.26	

specific gene-probe for mRNA-level gene-expression. ERCC1 and XPB expressions were previously determined by RT-PCR in 140 of tumor and normal specimens.

Real-time quantitative PCR. Real-time quantitative PCR (real-time qPCR) was performed for UBE2I, EGF, TAL2 and ILF3 using SYBR® Green reagents kit in the 131 samples. Amplifications were carried out on ABI PRISM 7000 Detection System and analyzed by ABI 7500 software (Applied Biosystems, Foster City, CA). In brief, all reactions were optimized to obtain the best amplification kinetics under the same cycling conditions (10 min at 95˚C, and 40 cycles of 15 sec at 95˚C and 1 min per cycle at 60˚C). Composition of the reaction mixture in a final volume of 20 μl containing Power SYBR Green Master Mix 10 μl; specific gene primers (10 μM) 1 μl; cDNA 2 μl. Negative controls that contain all PCR components without template DNA (denoted NTC) were used to ensure that the reagents mix were free of contamination. Each reaction was run in duplicate (8,18). The average threshold cycle (Ct) and the comparative ΔΔCt method were automatically calculated for the expression of the gene and normalized to mean Ct value of 18S ribosomal (endogenous control). The relative quantity (RQ) value was calculated using ΔΔCt method. Fold change in gene expression was calculated as $2^{(-\Delta\Delta Ct)}$. In this study, a gene Ct value of <32 was considered as positive expression and vice versa.

Statistical analysis. The gene expression data were summarized by mean, median (range) and standard error of the mean (SEM), and by box plot and scatter plot. The differences of gene expressions (ERCC1, XPB, TAL2, EGF, ILF3 and UBE2I) among tumor subtypes were examined by Kruskal-Wallis test, a non-parametric test based on Wilcoxon scores, followed by pairwise comparison between any two subtypes of ovarian cancer. The association between two gene expressions was estimated using Pearson correlation coefficient, and those found to be significant were summarized by scatter plot. The difference in gene expression levels between normal tissues and tumor tissues from the same patients was compared using paired t-test. Data from patients with complete pairs (normal and tumor) were used and statistical analyses were based on

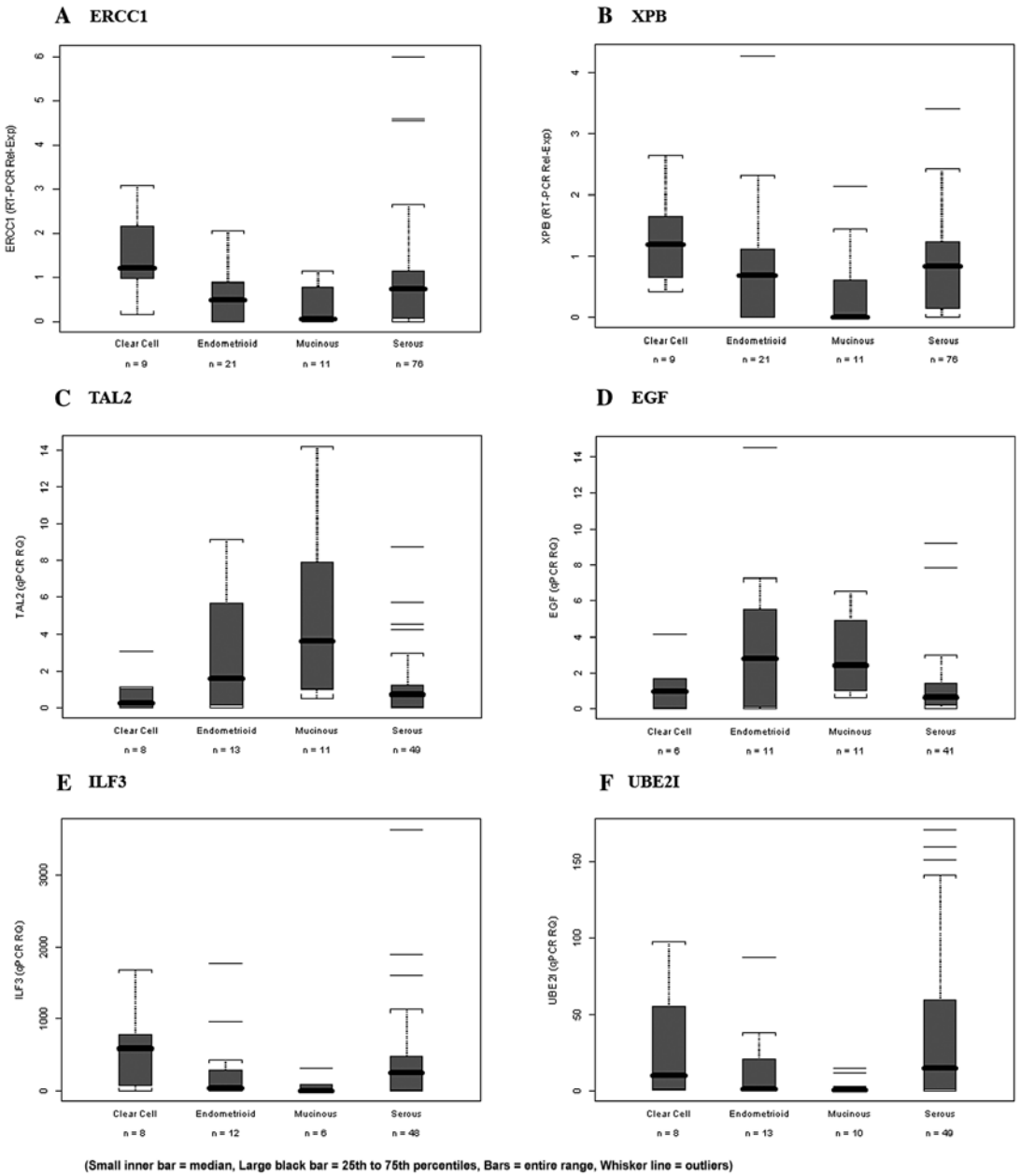

Figure 1. Box plots for ERCC1 (A), XPB (B), TAL2 (C), EGF (D), ILF3 (E) and UBE2I (F). Expressions in 4 subtypes of EOC. Pairwise comparison with p-value adjusted for only 2-comparison and estimated using Pearson correlation coefficient. Significant differences in each of the 6 genes (ERCC1, XPB, TAL2, EGF, ILF3 and UBE2I) were observed between any 2 subtypes of EOC.

complete cases without imput for the missing ones. All tests were two-sided and p-values ≤0.05 were considered statistically significant.

Results

Differential gene expression levels in tumor tissues, compared to normal ovarian samples. The expression data of the examined genes in studied specimens are summarized in Tables I and II. Table I shows the expression patterns of 6 genes in tumor tissues compared to normal samples. Collectively, the expression levels of ERCC1, XPB and ILF3 in tumor tissues were significantly higher than that in normal tissues (p=0.001, 0.0007 and 0.002, respectively). In contrast, the expression levels of TAL2 and EGF in tumor tissues were significantly lower than that in normal tissues (both p<0.0001). There was no statistical association of UBE2I expression between tumor and normal tissues (p=0.06).

Comparison of expression patterns between carcinomas and the corresponding normal ovarian tissues enabled us to identify 3 genes (ERCC1, XPB and ILF3) that were commonly up-regulated and 2 genes (TAL2 and EGF) that were down-regulated in these cancer patients. The differential expression patterns between normal and tumor tissues of ovarian carcinoma might reflect in part the cancer development of these patients.

Gene expression patterns among histological subtypes of epithelial ovarian cancer. Table II shows the expression results of the 6 genes in clear cell, endometrioid, mucinous and serous

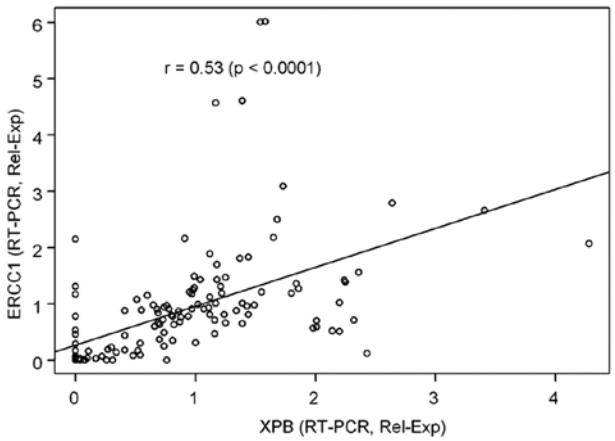

Figure 2. Significant association between ERCC1 and XPB expressions. Association of ERCC1 expression and XPB expression in tumor specimens estimated using Pearson correlation coefficient. This association is significantly positive with r=0.53 (p<0.0001).

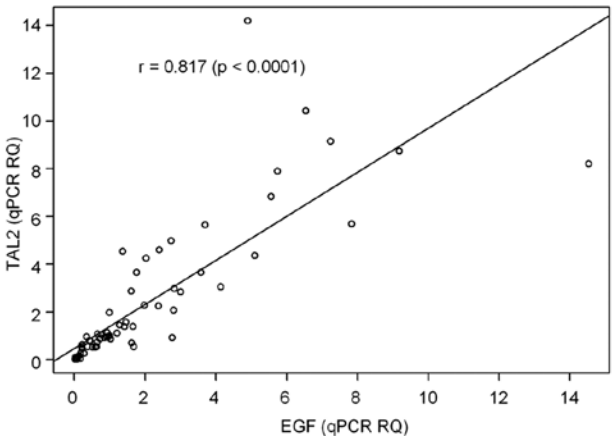

Figure 3. Significant association between TAL2 and EGF expressions. Association of TAL2 and EGF expressions in tumor specimens estimated using Pearson correlation coefficient based on complete data (some TAL2 and EGF expressions were missing for some subjects due to lack of adequate tissue samples). This significant association is strong and positive with r=0.817 (p<0.0001).

tumors. In brief, there were significant differences in expressions of ERCC1, XPB, TAL2, EGF and UBE2I among the 4 histological subtypes of ovarian carcinomas (p=0.004, 0.015, 0.003, 0.028 and 0.013, respectively). Note that the p-values cited here are for the comparison among 4 histological subtypes, not for comparisons between two tumor types. For ILF3, only a marginally significant difference (p=0.061) was observed among the 4 subtypes.

The difference in expression patterns of these genes among subtypes of epithelial ovarian cancer may arise from histopathologic differentiation and tumor progression in these patients.

The comparison of expression of any two EOC subtypes revealed multiple-gene involvement in histopathologic differentiation and cancer progression. A further pairwise comparison was performed with p-value adjusted for only 2-comparison and estimated using Pearson correlation coefficient (Fig. 1). We observed for ERCC1 (Fig. 1A), significant differences between i) clear cell and mucinous (p=0.002); ii) serous and mucinous (p=0.018); iii) clear cell and endometrioid (p=0.009); and iv) clear cell and serous (p=0.026). Fig. 1B shows significant differences of XPB expression between clear cell and mucinous (p=0.007) and between serous and mucinous (p=0.015). We also observed significant differences of TAL2 expression between clear cell and mucinous (p=0.003), and between serous and mucinous (p=0.0007) (Fig. 1C). For EGF, ILF3 and UBE2I expressions, there were significant differences between serous and mucinous (p=0.003) (Fig. 1D); between clear cell and mucinous (p=0.028); between serous and mucinous (p=0.035) (Fig. 1E); between endometrioid and serous (p=0.025); and between mucinous and serous (p=0.008) (Fig. 1F), respectively.

The significant differences of expression between any two subtypes of epithelial ovarian cancer indicate that multiple genes are activated as part of common signaling pathways in the cancer development. These genes are most likely involved in histopathologic differentiation and cancer progression.

Pearson correlation coefficient of expression demonstrated significant associations between ERCC1 and XPB and between TAL2 and EGF in measured tumor specimens. To further identify the possible linkage and relationship of any 2 genes we compared the corresponding results of gene expression using Pearson correlation coefficient (only the significant ones are summarized by scatter plot). As seen in Figs. 2 and 3, each dot represents the values of two genes. Thus, each point on these two figures is based on the values of both genes (i.e., x- and y-axis) and the correlation coefficient reflects an association between two genes. As shown in Fig. 2, there is an association between ERCC1 expression and XPB expression in measured tumor specimens. This association is positively significant with r=0.53 (p<0.0001). Fig. 3 shows a significant association between TAL2 and EGF expressions in tumor specimens. This association is strong and positive with r=0.817 (p<0.0001). Moreover, there were significantly negative associations between ERCC1 and EGF (r=-0.325; p=0.006); between XPB and TAL2 (r=-0.441; p=0.0001); and between XPB and EGF (r=-0.432; p=0.0002) (data not shown). The significant association of expression between two targeted genes suggests a linkage of these genes in EOC tumorigenesis and disease progression.

Discussion

Epithelial ovarian cancer is the most deadly malignancy among gynecological cancers. If diagnosed in stage I, up to 90% of patients can be cured with conventional surgery and chemotherapy, but current detection in stage I is only about 25%. Most patients present in an advanced stage. Detection of ovarian cancer in its early stages holds great promise for significantly improving the overall survival rate. To achieve this, a better understanding of the mechanism of tumorigenesis is critical. Given the heterogeneity of ovarian cancer, it is unlikely that a simple mechanism can explain tumor growth and refractory cases or that any single marker will be sufficiently sensitive for effective screening.

Investigators have found that the estrogen receptor (ER) is involved in tumor growth of several cancers. Some studies suggest that estrogens and their receptors play an important role in the growth and progression of colorectal tumors by interacting with growth factors (19); epidermal growth factor (EGF) couples ER-signaling pathway in the rodent reproductive tract (20). Other researchers report that EGF may induce c-fos mRNA and transactivation activity of AP1 via c-jun N-terminal kinase pathway (21). The post-translational modifications, ubiquitination and sumoylation have been implicated in regulating many critical cellular pathways. UBC9, encoded by UBE2I, is a sole E2-conjugating enzyme essential for sumoylation and regulates gene expression through ER-signaling pathway. In addition, loss of UBC9 function results in increased platinum sensitivity (22,23). Our early studies demonstrated that ERCC1 (excision repair cross-complementing 1, a key component of nucleotide excision repair pathway) and XPB (Xeroderma pigmentosum B, an ATP-dependent human DNA helicase) are up-regulated after cisplatin treatment. AP1, the promoter-activator, is required for ERCC1 transcription and expression by forming a complex (c-jun homodimer or c-jun/c-fos heterodimer), resulting in the removal of cisplatin-induced DNA-adduct damage and leading to platinum-resistance and treatment failure (24-28). The involvement of multiple genes in advanced ovarian cancer was suggested by our primary gene signature study (8). The correlations among involved genes of EOC histological subtypes are further revealed by this investigation. These data indicate a mechanism by which panels of up-regulated and down-regulated genes contribute to the development and progression of refractory tumors in EOC patients. Very likely, multiple genes play different roles in the complicated etiological evolution of this disease.

In addition, it is known that ERCC1 and XPB contribute to clinical response by increasing DNA repair activity and reducing apoptosis, which is the leading mechanism of drug resistance in platinum-chemotherapy (6,28,29). Other researchers have observed the linkage between studied genes and drug responsiveness, including lack of UBE2I function in increased cisplatin sensitivity; EGF-induced AP1 activation/transcription leads to increased c-fos transactivation through the MAPK pathway (21,22). Our previous investigation demonstrated that ERCC1 overexpression, mediated by its promoter-activator AP1 (c-fos/c-jun), is associated with repair of cisplatin-induced DNA-adduct and represents the platinum-resistant phenotype (24-26). ERCC1 expression is elevated in tissues from patients refractory to cisplatin treatment and reflects DNA repair capacity and clinical resistance to platinum chemotherapy. In our current study, significant correlation of expression between EGF and ERCC1, between ERCC1 and XPB in tumor tissues, may be useful guides for treatment. For example, ERCC1 and XPB are both highly involved in platinum-drug resistance and the DNA-damage/repair pathway. Thus, the association of these two genes and their expression levels may predict clinical response to the platinum therapy for these patients.

Acknowledgements

The Molecular Medicine Core Facility, Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV performed the laboratory analyses reported herein.

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