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Ubiquitin–Proteasome Axis, Especially Ubiquitin-Specific Protease-17 (*USP17*) Gene Family, is a Potential Target for Epithelial–Mesenchymal Transition in High-Grade Serous Ovarian Cancer

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Abstract

Objectives: To investigate gene expression differences and related functions between primary tumor, malignant cells in ascites, and metastatic peritoneal implant in high-grade serous ovarian cancer. **Methods:** Biopsies from primary tumor, peritoneal implant, and ascites were collected from 10 patients operated primarily for high-grade, advanced-staged serous ovarian cancer. Total RNA isolation was performed from collected tissue biopsy and fluid samples, and RNA expression profile was measured. Messenger RNA expression profiles of 3 different groups were compared. Functional analyses of candidate genes were carried out by gene ontology and pathway analysis. **Results:** There were significant differences in the expression of 5 genes between primary tumor and peritoneal implant, 979 genes between primary tumor and malignant cells in ascites, and 649 genes between peritoneal implant and malignant cells in ascites. Three commonly enriched gene ontology functions between "primary tumor and malignant cells in the ascites" and "peritoneal implant and malignant cells in the ascites" were protein deubiquitination, ubiquitin-dependent protein catabolism, and apoptotic processes. All genes related to these functions belonged to *USP17* gene family. **Conclusion:** Gene expression difference between primary tumor and the peritoneal implant is not as much as the difference between primary tumor and free cells in the ascites. These results show that malignant cells in the ascites return into its genetic origin after they invade on the peritoneum. Significantly increased expression of DUB-enzyme genes, *SNAR* gene family, and ribosomal pathway genes in epithelial–mesenchymal transition suggests that this regulation is ubiquitin–proteasome dependent. Especially, this is the first study that offers *USP17* as a potential target for epithelial–mesenchymal transition.

Keywords

ovarian cancer, serous, microarray, epithelial–mesenchymal transition, deubiquitination, ubiquitin–proteasome pathway

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Introduction

Ovarian cancer is the major cause of death among gynecologic cancers.[1] It has many histologic subtypes, but serous ovarian cancers are the most common subtype. Serous ovarian tumors have 2 types: low grade and high grade. High-grade serous ovarian tumors are classified under type 2 ovarian cancer according to the hypothesis about pathogenesis of ovarian cancer.[2] Type 2 cancer is more aggressive than type 1 (eg, low-grade serous tumor), mostly metastatic at diagnosis and generally has p53 mutations.

The main treatment strategy is primary surgery and adjuvant chemotherapy, and the main goal during surgery is to achieve zero macroscopic disease.[3] Patient whose medical condition is not suitable for primary surgery or who is not a good candidate for optimal cytoreduction may receive neoadjuvant chemotherapy followed by interval debulking surgery.[4] Targeted therapy is one of the most popular topics in cancer research, and in ovarian cancer, there are some approved or experimental drugs. Bevacizumab, vascular endothelial growth factor monoclonal antibody, is the most well-known drug for targeted therapy in ovarian cancer.

Although surgical approach has had a great improvement since last 15 years regarding radicality and new evidence-based medical treatment strategies were introduced, mortality has not changed significantly and patients still die due to complications of recurrent/metastatic disease. Mortality rates declined only slightly in the last 40 years and have been falling on average 2.2% each year between 2005 and 2014.[5] There are 2 ways to decrease mortality rates. The first one is to prevent disease, such as cervical cancer. But heretofore, there has not been any effective screening method in ovarian cancer. The other way is to treat the disease with exact cure. The second seems more difficult than the first one, because, especially for high-grade serous tumors, nearly 75% of patients are at advanced stages at the time of diagnosis.[6] The other point is that although the patients were treated optimally and had remission, nearly half of them had relapse in a 2-year period.[7] Unfortunately, 5-year overall survival rates were 46.5%.[5]

Major problem in the treatment is metastatic nature of the disease, since it tends to metastasize both at the time of diagnosis and after first-line treatment. Little is known about the metastasis of ovarian cancer, but recent studies introduced more about the metastasis pathophysiology of epithelial tumors. Epithelial–mesenchymal transition (EMT) and the opposite of this process, mesenchymal–epithelial transition (MET), are critical steps in that field. Epithelial–mesenchymal transition can be defined as switch of epithelial cell into mesenchymal cells by weakening of cell–cell interactions in the epithelium after multiple biochemical changes, with enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis.[8] In this complex transition, many biologic processes take part such as activation of transcription factors, rearrangement of cytoskeletal proteins, production of specific cell-surface proteins/extracellular matrix enzymes, and changes in the expression of specific genes or microRNAs.[9]

The way of intraperitoneal spread of high-grade serous ovarian tumor seems theoretically compatible with EMT and MET, because, after capsule invasion of tumor cells, they pass into ascites, move freely in the abdomen with the help of fluid flow, and then invade peritoneal surfaces. In this study, we aimed to investigate the changes in gene expression between 3 groups of cells, namely, primary tumor cells, malignant cells in the ascites, and peritoneal implant cells of high-grade serous ovarian tumors in order to understand the gene expression changes in different steps of EMT. Up to now, there are limited number of studies about transgenomic gene expression changes in ovarian cancer, but nearly all of them compared the changes between normal ovarian cells and primary tumor or primary tumor and metastatic implant.[10-13] This is the first study that analyses transgenomic gene expression changes in 3 different groups of cells in high-grade serous ovarian tumor.

Materials and Methods

Patient Selection and Sample Collection

In this prospective study, patients who were operated primarily for high-grade advanced stage (International Federation of Gynecology and Obstetrics [FIGO] stage 3-4) serous ovarian cancer between 2015 and 2017 were included. Patients who were FIGO stage 1-2 and who had neoadjuvant chemotherapy were not included. Patients with final pathology reports other than serous papillary carcinoma (other epithelial, germ cell, sex-cord stromal, and metastatic) were excluded from the study. The study was evaluated and approved by the Ethics Committee of Dokuz Eylul University (protocol number: 2014/01-16) in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients.

After midline vertical incision, 100 cm^3 ascites was taken from each patient. Then, at least 1 cm^3 tumor samples, which were histopathologically proven during frozen section, were obtained from both primary ovarian tumor and peritoneum preferably at right paracolic region outside the pelvis. Specific care was taken not to have peritoneal biopsy adherent to the pelvic mass. Fresh tissue samples were stored directly at −80 C. In order to get malignant cells in the ascites, it was centrifuged at 1200 rpm for 5 minutes. After centrifugation, precipitate was divided into 2. Cytospin technique was performed to half of the precipitate to immobilize the cells on the slides.[14] After routine hemotoxylin and eosin staining, slides that include more than 70% malignant cells were included for further microarray analysis.[15] Patients whose cytological evaluation did not meet the criteria were excluded from the study.

Immunohistochemistry

Immunohistochemistry was used to detect and quantify E-cadherin and N-cadherin expression for the confirmation of epithelial and mesenchymal phenotypes from the formalin-fixed paraffin-embedded tissue/cell sections of representative primary tumor, cells from ascites, and peritoneal implant

samples. Separate slides were treated with biotinylated anti-E-cadherin (Cell Signaling Technology, Massachusetts) and N-cadherin (Dako, Glostrup, Denmark) primary antibodies following manufacturer's instructions. Staining was performed using automatic immunohistochemistry staining equipment (lab vision autostainer; Thermo Scientific, USA) and evaluated by pathologists. Diffuse- or focal-stained specimens were accepted as positive.

RNA Isolation

RNA isolation was performed using RNeasy mini kit (Qiagen, Valencia, California). Malignant cells from ascites and tissue samples were lysated in buffer RLT and followed all steps as recommended by the manufacturer's instructions. The total RNA concentration was determined by a nanodrop spectrophotometer (NanoDrop Technologies Inc, Montchanin, Delaware).

Microarray Hybridization

After total RNA isolation, genome-wide transcriptome analysis was performed using the GeneChip Human Gene 2.0 ST array that contained probe sets covering all exons of $\sim$33 500 coding transcripts of annotated genes. According to the manufacturer's instructions, RNA samples were processed using Affymetrix GeneChip WT terminal labeling and hybridization, wash, and stain kits (Affymetrix, Inc, Santa Clara, CA, USA). Briefly, single-strand complementary DNA was fragmented with a specific enzyme cocktail and labeled by terminal deoxynucleotidyl transferase with the DNA labeling reagent. After labeling process, coding RNA (cRNA) was hybridized on GeneChip Human Gene 2.0 ST arrays. Array slides were washed and stained (streptavidin and biotin based) in the Affymetrix Fluidics Station 400 (Affymetrix, Inc) and scanned with Affymetrix scanner 3000 7G (Affymetrix, Inc).

Microarray Data Processing

Raw data were processed with the Command Console Software 4.0 (Affymetrix, Inc) using default settings and the appropriate quality control. Data were normalized and then $\log_2$-transformed. The signals were accepted as the quantity of messenger RNA (mRNA) existence derived from the level of gene expression. Obtained data were standardized using a z-score calculation.

Determination of Differentially Expressed Genes and Hierarchical Clustering Analysis

Differentially expressed genes among different groups were identified using the Linear Models for Microarray data package in R-Bioconductor, and fold-changes ($\log_2$FC) were calculated. As a threshold, $|\log_2 FC| \geq 2$ and adjusted P value $\leq .05$ were used for identifying differentially expressed genes.

Heatmaps were drawn to show the differences between 2 samples. The z-score was calculated for data normalization, and each gene was normalized using the sample mean and standard deviation values. So, the z-score of a gene shows the normalized expression value of gene for the experiment sample (eg, primary tumor). If annotation of a transcript identifier were mapped to more than 1 gene name by the manufacturer, we could not display several gene names of such transcripts. Hence, we provided all annotated gene names of these transcript identifiers in Supplemental data. Heatmaps were plotted using a hierarchical clustering method provided in the Heatplus library of R-Bioconductor. A filtering applied to choose transcripts whose z-scores are on the opposite ends of the normal distribution, that is, select a transcript if its z-score is positive in one sample and negative in another. Only these filtered and significant transcripts were plotted in the final heatmaps.

Biological Function Analysis of Differentially Expressed Genes

A functional enrichment analysis was applied to determine the biological processes and signaling pathways of statistically significant genes. For this purpose, the genes with the high FC values were given as an input data to the DAVID website.[16] As a result of analysis, the gene ontology (GO) terms (only those have biological process labels) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, which were smaller than corrected P value (Bonferroni) of .05, were considered as significant.

Network-Based Functional Analysis

A network-based analysis was applied to understand functions of significant genes in a protein–protein interaction (PPI) network. The STRING network was used for this analysis.[17] The significant genes plotted in the heatmaps were selected and searched over a high-scored (interaction score ≥ 0.9) STRING PPI network. Most of the significant genes were not retrieved in this search due to high confidence of interactions in the STRING network. Therefore, the available significant genes were connected via other genes in the network. Two subnetworks were constructed for peritoneal implant versus ascites tumor cells and primary tumor versus ascites tumor cells. A second functional enrichment analysis was performed to learn GO annotations and KEGG pathways of the genes that are only covered in the subnetworks.

Results

After exclusion of patients whose final pathology reports were other than serous carcinoma and whose cytological specimens did not contain enough malignant cells, 10 patients were included in this study. Histopathological and demographic features are summarized in Table 1.

Table 1. Demographic and Histopathological Features of Patients.

Patient Number	Age	CA125 (U/mL)	Histology	FIGO Stage	Histologic Grade
1	36	648,5	Serous	4	3
2	54	6123	Serous	3c	3
3	54	253	Serous	3c	3
4	55	89	Serous	3c	3
5	37	1851	Serous	3c	3
6	57	5363	Serous	3c	3
7	45	666	Serous	4	3
8	67	647	Serous	3c	3
9	45	3483	Serous	3c	3
10	60	3218	Serous	3c	3

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

Transcriptomic gene expression changes were evaluated in 3 groups of cells in these 10 patients. These groups of cells were primary ovarian tumor cells, peritoneal implant cells, and malignant cells in the ascites. Gene expression changes between the groups were compared 2-by-2.

Immunohistochemistry

Representative primary tumor, cells from ascites, and peritoneal implant samples were stained with EMT markers E-cadherin and N-cadherin for the confirmation of epithelial and mesenchymal phenotypes (Figure 1). E-cadherin expression was significantly decreased in malignant cells in the ascites compared to primary tumors and peritoneal implant ($*P < .05$). N-cadherin expression was significantly increased in malignant cells in the ascites compared to primary tumors and peritoneal implant ($*P < .05$). According to the immunohistochemistry results, decreased E-cadherin expression and increased N-cadherin expression are accepted as an indicator of increased mesenchymal phenotype in malignant cells in the ascites.

Microarray Results

Five genes were significantly differently expressed between primary tumor and peritoneal implant. *RNU6-135P*, *RNU6-1262P*, and *VTRNA1-1* genes were upregulated in primary tumor; *MCAM* and genes belonging to *SNAR* gene family were upregulated in peritoneal implant (Figure 2A, Supplemental Table 1).

The number of differentially expressed genes between primary tumor and malignant cells in the ascites is 978. From 978 differentially expressed genes, 762 of them were upregulated in primary tumor and the rest 216 were upregulated in free malignant cells (Supplemental Table 2). Between malignant cells in the ascites and peritoneal tumoral implant, 648 genes were differentially expressed, and 515 of them were upregulated in peritoneal implant where 133 of them were upregulated in the malignant cells in ascites (Supplemental Table 3). Hierarchical clustering plots of primary tumor versus malignant cells in the ascites and malignant cells in the ascites versus peritoneal tumoral implant were given in Figure 2.

Gene Ontology Terms and KEGG Pathways

The enriched GO functions with significantly corrected *P* and Bonferroni values for the candidate genes were summarized in Table 2. Since there were only 5 differentially expressed genes between primary tumor and peritoneal implant, no enriched GO functions were detected in this group. Three common GO-enriched processes were observed between the comparisons of other groups, which are "primary tumor versus malignant cells in the ascites" and "malignant cells in the ascites versus peritoneal implant." They were protein deubiquitination (GO:0016579), ubiquitin-dependent protein catabolism process (GO:0006511), and apoptotic process (GO:0006915). All of 27 genes related to protein deubiquitination, all of 26 genes related to ubiquitin-dependent protein catabolism process, and 11 of 12 genes related to apoptotic process belonged to *USP17* gene family. Other significantly enriched GO functions between primary tumor versus malignant cells in the ascites were SRP-dependent cotranslational protein targeting to membrane (GO:0006614), nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (GO:0000184), translational initiation (GO:0006413), and ribosomal RNA processing (GO:0006364; Table 2).

The enriched KEGG pathways for the candidate genes were listed in Table 3. Two significantly enriched KEGG pathways between primary tumor and malignant cells in the ascites were "ribosome (hsa03010)" and "ribosome biogenesis in eukaryotes (hsa03008)." Six genes (*RPS3A*, *RPL13A*, *RPL23A*, *RPL7A*, *RPS2*, and *RPS8*) related to "ribosome pathway" were upregulated in primary tumor, whereas 4 genes (*LOC101929601*, *LOC100288562*, *LOC101929627*, and *LOC101930111*) related to "ribosome biogenesis in eukaryotes pathway" were upregulated in free malignant cells in ascites. There was no significantly enriched KEGG pathway between malignant cells in the ascites and peritoneal implant (Figures 3 and 4).

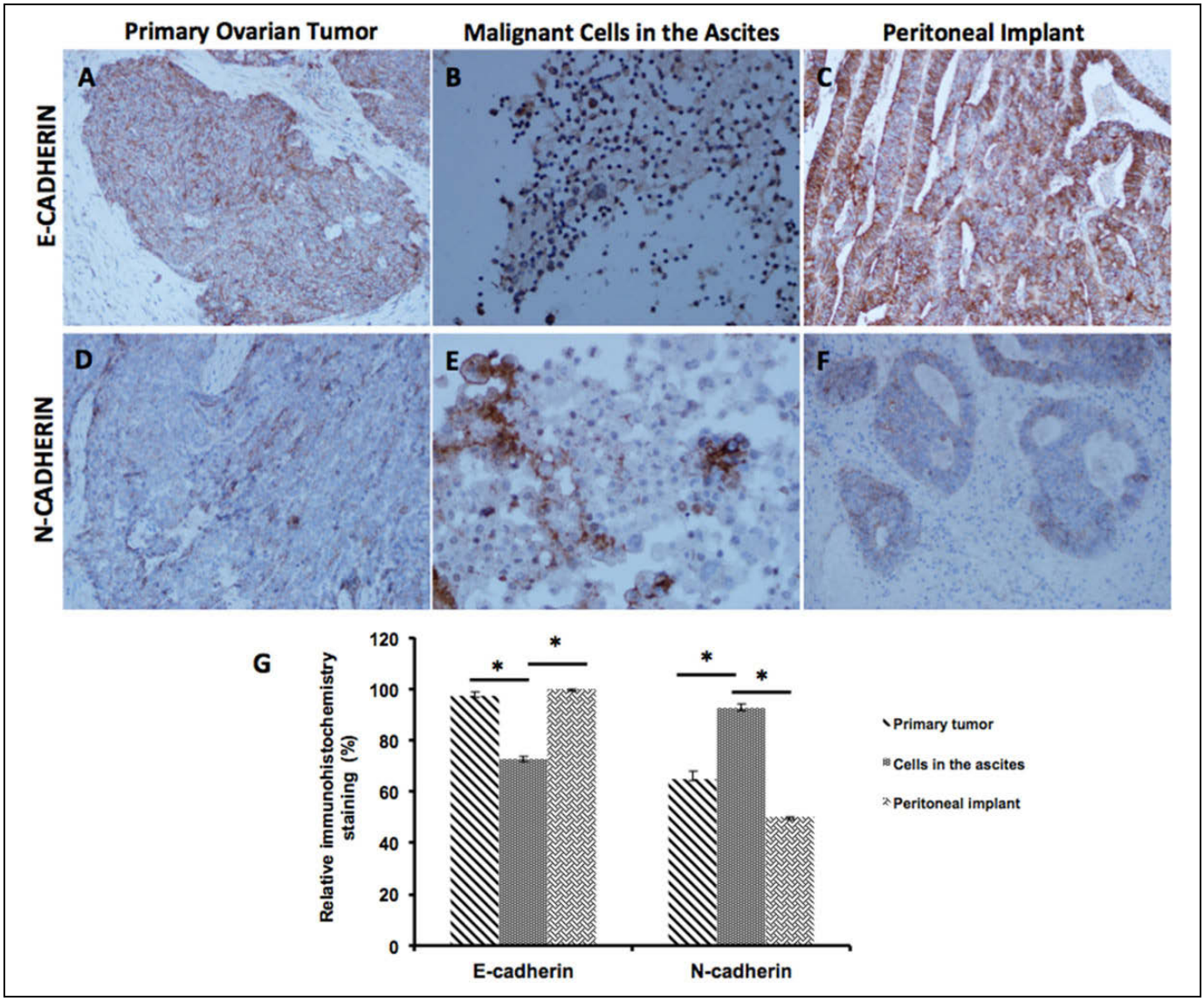


Figure 1. Immunohistochemical staining of E-cadherin (epithelial) and N-cadherin (mesenchymal) markers of representative primary tumor, malignant cells in the ascites, and peritoneal implant samples. (A) E-cadherin staining of primary tumor (magnification ×20), (B) E-cadherin staining of malignant cells in the ascites (magnification ×40), (C) E-cadherin staining of peritoneal implant (magnification ×20), (D) N-cadherin staining of primary tumor (magnification ×20), (E) E-cadherin staining of malignant cells in the ascite (magnification ×40), (F) E-cadherin staining of peritoneal implant (magnification ×20), and (G) the quantitative of relative percentage of positive E-cadherin and N-cadherin staining. *P 0.05.

Discussion

Since the most challenging part of ovarian cancer management is metastasis and dense seeding of the peritoneal cavity by malignant cells is often related to ascites in high-grade serous carcinomas,[18] this study was designed to understand the transcriptomic differences in this spreading route using tissues or cell samples from primary ovarian tumor, malignant cells in the ascites, and peritoneal implants. At the beginning process of metastasis, malignant cells switch their phenotype from epithelial to mesenchymal with the decreased expression of epithelial molecules and increased expression of mesenchymal molecules. Accompanied to these alterations, malignant cells lose their contacts with neighboring tissue, gain motile characteristics, and enter the fluidic circulation. Therefore, we hypothesized that primary ovarian tumors represent epithelial phenotype, malignant cells in the ascites represent gained mesenchymal phenotype with EMT-based alterations, and peritoneal implants represent regained epithelial character with MET. In this study, we investigated the transcriptomic gene expression changes between primary tumor, malignant cells in the ascites, and peritoneal tumoral implant for the first time, in order to determine the candidate genes that play role in different steps of metastasis.

Previous genome-wide studies about ovarian cancer had insufficient design to describe candidate genes for metastatic pathways because they compared "primary tumor and

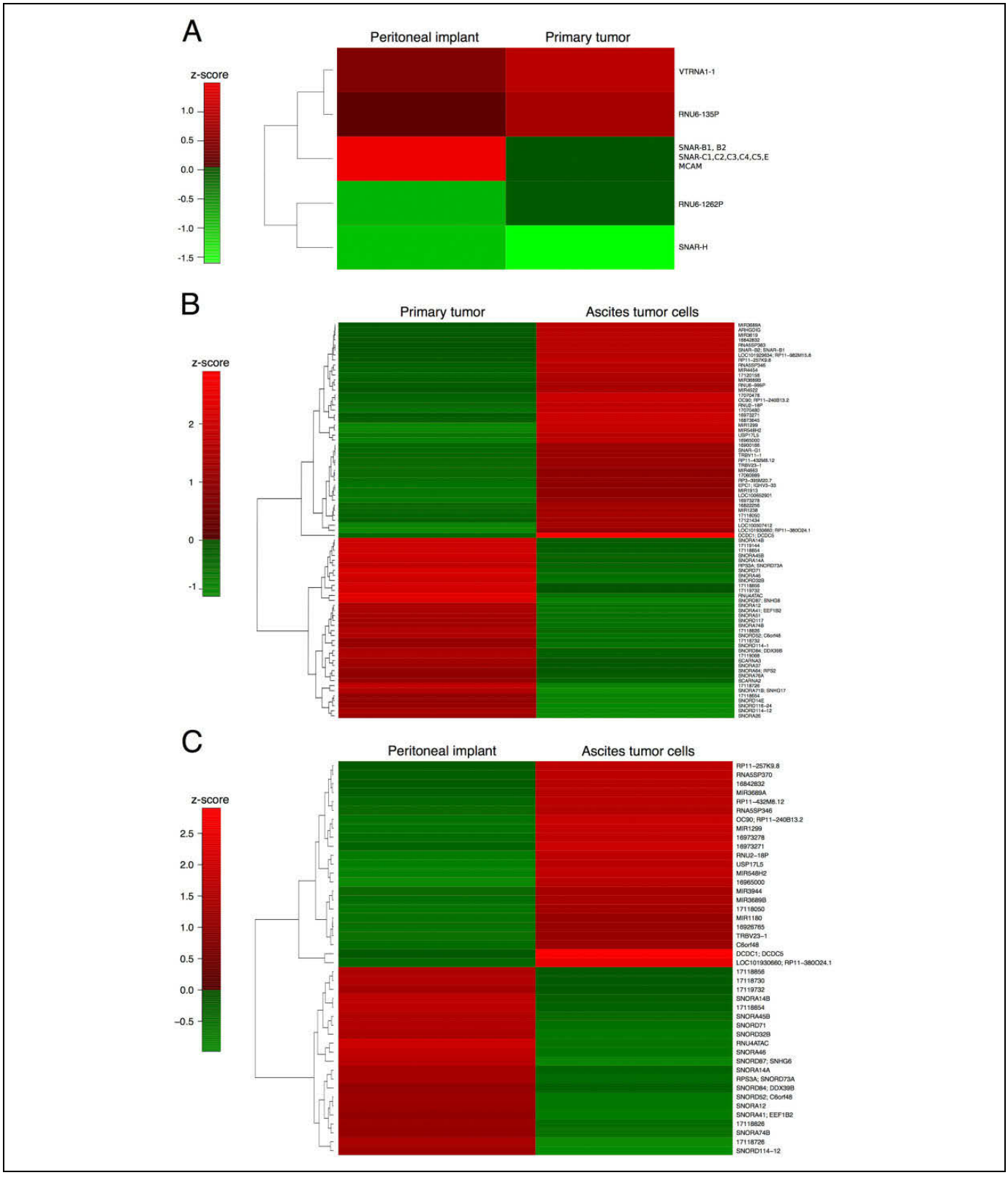


Figure 2. A, Hierarchical clustering plot of primary tumor versus peritoneal implant (Supplemental Table 1). B, Hierarchical clustering plot of primary tumor versus malignant cells in the ascites. A subset of differential genes (n = 79) was selected from all differentially expressed genes (n = 978) between primary tumor and malignant ascites cells in for clustering analysis (Supplemental Table 2). C, Hierarchical clustering plot of malignant cells in the ascites versus peritoneal tumoral implant. A subset of differential genes (n = 44) was selected from all differentially expressed genes (n = 648) between malignant ascites cells and peritoneal implant for clustering analysis (Supplemental Table 3). Clustering analysis was performed as described in the "Material and Method" section. Upregulated and downregulated genes are represented in red and green colors, respectively. Transcript cluster IDs were used for the determination of gene names; transcript ID/gene name list were given in Supplemental Table 4.

Table 2. GO Terms Significantly Enriched in the Differentially Expressed Genes (for Both "Primary Tumor Versus Malignant Cells in Ascites" and "Peritoneal Implant Versus Malignant Cells in Ascites").

Term	Genes	Count	*P* Value	Bonferroni
Primary tumor versus malignant cells in ascites				
GO:0016579 Protein deubiquitination	*USP17L6P*, *USP17L27*, *USP17L26*, *USP17L29*, *USP17L28*, *USP17L23*, *USP17L22*, *USP17L25*, *USP17L24*, *USP17L3*, *USP17L21*, *USP17L20*, *USP17L2*, *USP17L9P*, *USP17L19*, *USP17L30*, *USP17L18*, *USP17L17*, *USP17L15*, *USP17L13*, *USP17L12*, *USP17L11*, *USP17L10*, *USP17L8*, *USP17L7*, *USP17L5*, *USP17L4*	27	1.74E−44	2.00E−42
GO:0006511 Ubiquitin-dependent protein catabolic process	*USP17L9P*, *USP17L6P*, *USP17L30*, *USP17L19*, *USP17L18*, *USP17L27*, *USP17L26*, *USP17L17*, *USP17L29*, *USP17L28*, *USP17L15*, *USP17L23*, *USP17L22*, *USP17L13*, *USP17L12*, *USP17L25*, *USP17L24*, *USP17L11*, *USP17L10*, *USP17L21*, *USP17L3*, *USP17L2*, *USP17L20*, *USP17L8*, *USP17L7*, *USP17L5*	26	5.64E−35	6.48E−33
GO:0006915 Apoptotic process	*USP17L9P*, *USP17L30*, *USP17L27*, *USP17L26*, *USP17L29*, *USP17L28*, *USP17L25*, *USP17L24*, *DUX4*, *USP17L5*, *USP17L3, USP17L2*	12	2.21E−06	2.54E−04
GO:0006614 SRP-dependent cotranslational protein targeting to membrane	*RPS3A*, *RPL13A*, *RPL23A*, *RPL7A*, *RPS2*, *RPS8*	6	1.51E−05	0.001729479
GO:0000184 Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	*RPS3A*, *RPL13A*, *RPL23A*, *RPL7A*, *RPS2*, *RPS8*	6	4.70E−05	0.00539209
GO:0006413 Translational initiation	*RPS3A*, *RPL13A*, *RPL23A*, *RPL7A*, *RPS2*, *RPS8*	6	9.19E−05	0.010511638
GO:0006364 rRNA processing	*RPS3A*, *RPL13A*, *RPL23A*, *RPL7A*, *RPS2*, *RPS8*	6	7.23E−04	0.07977371
Peritoneal implant versus. malignant cells in ascites				
GO:0016579 Protein deubiquitination	*USP17L6P*, *USP17L27*, *USP17L26*, *USP17L29*, *USP17L28*, *USP17L23*, *USP17L22*, *USP17L25*, *USP17L24*, *USP17L3*, *USP17L21*, *USP17L20*, *USP17L2*, *USP17L9P*, *USP17L19*, *USP17L30*, *USP17L18*, *USP17L17*, *USP17L15*, *USP17L13*, *USP17L12*, *USP17L11*, *USP17L10*, *USP17L8*, *USP17L7*, *USP17L5*, *USP17L4*	27	4.64E−49	2.97E−47
GO:0006511 Ubiquitin-dependent protein catabolic process	*USP17L9P*, *USP17L6P*, *USP17L30*, *USP17L19*, *USP17L18*, *USP17L27*, *USP17L26*, *USP17L17*, *USP17L29*, *USP17L28*, *USP17L15*, *USP17L23*, *USP17L22*, *USP17L13*, *USP17L12*, *USP17L25*, *USP17L24*, *USP17L11*, *USP17L10*, *USP17L21*, *USP17L3*, *USP17L2*, *USP17L20*, *USP17L8*, *USP17L7*, *USP17L5*	26	2.94E−39	1.88E−37
GO:0006915 Apoptotic process	*USP17L9P*, *USP17L30*, *USP17L27*, *USP17L26*, *USP17L29*, *USP17L28*, *USP17L25*, *USP17L24*, *DUX4*, *USP17L5*, *USP17L3*, *USP17L2*	12	9.77E−08	6.25E−06

Abbreviations: GO, gene ontology; rRNA, ribosomal RNA.

Table 3. KEGG Pathways Significantly Enriched in the Differentially Expressed Genes of Primary Tumor and Malignant Cells in Ascites.

Term	Genes	Count	*P* Value	Bonferroni
Primary tumor versus malignant cells in ascites				
hsa03010: Ribosome	*RPS3A*, *RPL13A*, *RPL23A*, *RPL7A*, *RPS2*, *RPS8*	6	4.76E−06	4.76E−05
hsa03008: Ribosome biogenesis in eukaryotes	*LOC101929601*, *LOC100288562*, *LOC101929627*, *LOC101930111*	4	6.35E−04	0.006329782

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

peritoneal implant" or "normal ovarian tissue and primary tumor."[10-13] Additionally, some of them included different histologic subtypes that could totally alter the results. Our study demonstrated the candidate genes that were responsible in different steps of metastasis in a homogeneous group of patients.

In a previous study, Yoshida et al reported that 108 genes related to EMT and 46 genes unrelated to EMT were significantly differentially expressed between primary tumor and omental implant.[10] *AKT*, *ANXA*, *ANGII*, *AXI2*, *BCL2*, and *BCL-XL* related to intracellular signal transduction; *ANGII*, *BMP7*, *MET*, *EGF*, *ENG*, *MAPK*, and *EPO* related to growth factor synthesis; *ZEB1*, *TCF3*, and *HEY1* related to transcription factors; and *FLNA*, *ICAM1*, *CDH1*, *CDH2*, *CDH3*, *TLN1*,

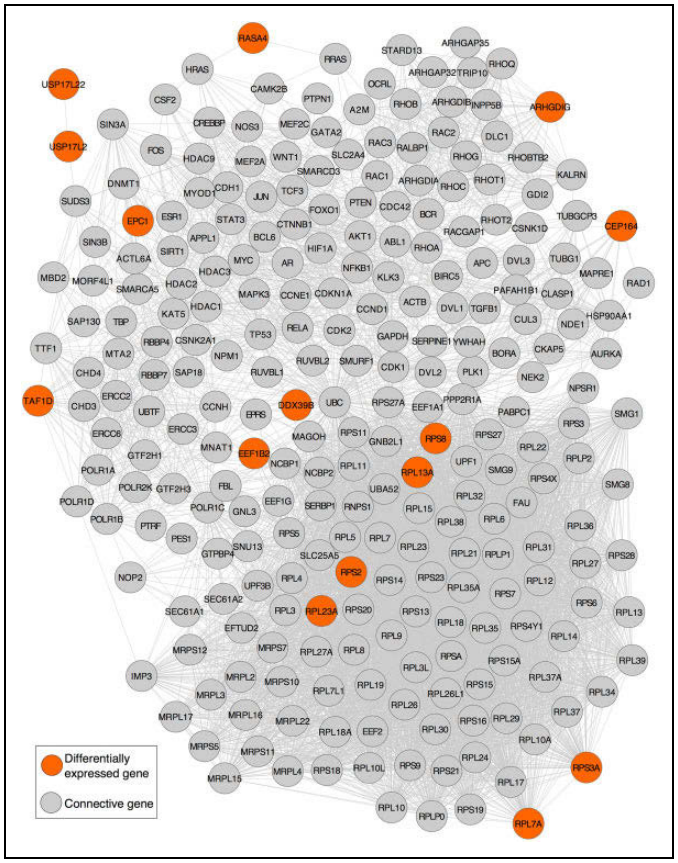


Figure 3. Protein–protein interaction network of the differentially expressed genes between primary tumor and malignant cells in the ascites.

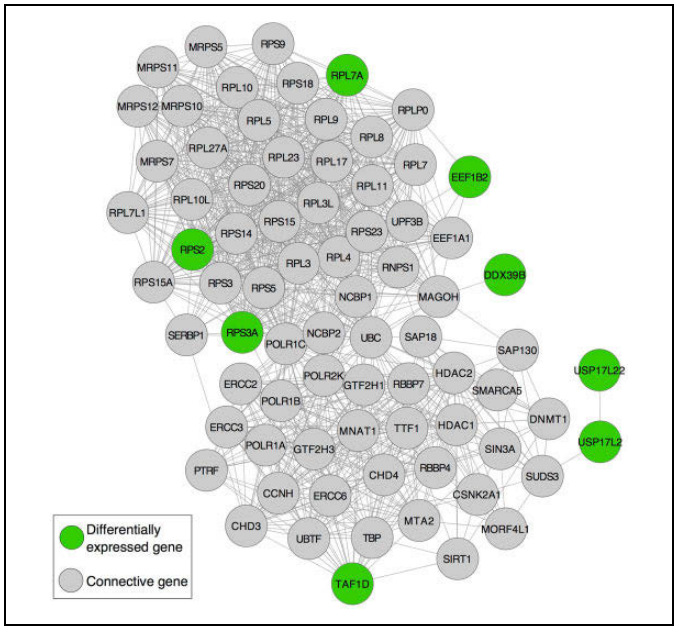


Figure 4. Protein–protein interaction network of the differentially expressed genes between peritoneal implant and malignant cells in the ascites.

and *VIM* related to cell adhesion were the examples of EMT-associated genes.

In our study, representative primary tumor, malignant cells from ascites, and peritoneal implant samples were stained with EMT markers such as E-cadherin and N-cadherin for the confirmation of epithelial and mesenchymal phenotypes. E-cadherin is used as an epithelial and N-cadherin as a mesenchymal marker (Figure 1). According to the results, E-cadherin expression was significantly decreased in malignant cells in the ascites compared to primary tumors and peritoneal implant (*$P < .05$), and N-cadherin expression was significantly increased in malignant cells in the cells compared to primary tumor and peritoneal implant (*$P < .05$). Appearance of decreased E-cadherin expression and increased N-cadherin expression at the same time is accepted as E/N-cadherin switch, an indicator of increased mesenchymal phenotype. "E/N-cadherin switch" is required for the phenotypic switch during EMT process; therefore, this alteration promotes cancer progression and metastasis. Our results present the existence of phenotypic switch from epithelial to mesenchymal by E/N-cadherin switch, and these alterations are indicators of the EMT.

Also, the network-based functional analysis was carried out to observe the relationship between differentially expressed genes and EMT-effectors/regulators due to the EMT process. *E-cadherin (CDH1)*, *EpCAM*, *Vimentin (VIM)*, and *N-cadherin (CDH2)* were used as EMT-effectors; and SNAI1 (SNAIL), SNAI2 (SLUG), SNAI3 (SMUC), ZEB1, ZEB2, TWIST1, TWIST2, and TGF-β were used as EMT regulators.[19-21] Network analysis showed that differentially expressed genes between "primary tumor and malignant cells in the ascites" and "peritoneal implant and malignant cells in the ascites" were linked to *E-cadherin (CDH1)*, *Vimentin (VIM)*, *N-cadherin (CDH2)*, SNAI1, SNAI2, and TGFB, which are the main EMT-effectors/regulators (Supplemental Figures 1 and 2).

In our study, genes that were differentially expressed between primary tumor and peritoneal implant were *RNU6-135P*, *RNU6-1262P*, *VTRNA1-1*, *MCAM* and some of the genes that belong to *SNAR* gene family (*SNAR-B1*, *SNAR-B2*, *SNAR-C1*, *SNAR-C2*, *SNAR-C3*, *SNAR-C4*, *SNAR-C5*, *SNAR-E*, and *SNAR-H*; Supplemental Table 1).

Long noncoding RNAs (lncRNA) are involved in heterogeneous class of transcript, which is composed of more than 200 nucleotides. The lncRNAs regulate gene expression by various mechanisms that are not yet fully understood. Alterations in the expression levels of numerous lncRNAs have been determined in various cancers, and the roles and mechanisms of some of them have been identified as well.[22] Small NF90-associated RNAs (SNAR) are highly structured lncRNAs and they bind to nuclear factor 90 (NF90), a mammalian double-stranded RNA-binding protein encoded by the *ILF3* gene.[23] In humans, SNAR species were called as SNAR-A, SNAR-B, *SNAR-C*, *SNAR-D*, *SNAR-E*, *SNAR-F*, *SNAR-G1*, *SNAR-G2*, *SNAR-H*, *SNAR-I*, *SNAR-12*, and *SNAR-21*; and there are nearly 30 *snaR* genes included to these species. SNAR-A, SNAR-B, *SNAR-C*, and *SNAR-D* members that accounting for about half of the

SNARs are clustered on the q-arm of chromosome 19 in 2 tandem sequences. *SNAR-H*, *SNAR-I*, *SNAR-12,* and *SNAR-21* are placed on chromosomes 2, 3, 12, and 21, respectively.[24]

In various breast cancer cell lines, SNAR upregulation was demonstrated. In a study carried out by Lee et al to understand the role of lncRNAs in breast cancer cell lines, the upregulation of SNAR in MDA-MD-231 and MCF7 cell lines was observed, and the downregulation of SNAR inhibited the proliferation, migration, and invasion capacity of MDA-MD-231 breast cancer cells. Therefore, SNAR knockdown can be a promising modulation for triple-negative breast cancer treatment.[25] In another study carried out by the same group, SNAR upregulation was identified in HER2-positive breast cancer cell lines (MCF7, SK-BR3, T47D, and BT474) and concluded that the study with the same approach such as knockdown of SNAR was strongly associated with suppression of tumor proliferation and migration.[23] In our study, *SNAR* genes on both chromosomes 19 and 2 were upregulated in peritoneal implant when compared to primary tumor. According to this, SNARs can have role on the migration and invasion capacity of the ovarian cancer.

Another gene family related to the ribosome is U6 RNA, and it has been reported that tissue distributions in this wide variety of cancers differ.[26] In our group, RNU6-135P (RNA, U6 small nuclear 135, pseudogene) and RNU6-1262P were downregulated in peritoneal implant when compared to primary tumor. RNU6-135P and RNU6-1262P are pseudogenes, which are nonfunctional fragments of the DNA. But despite being noncoding regions, most of the pseudogenes have significant roles in physiological or pathological processes.[27] But the function of RNU6-135P and RNU6-1262P is not known in the literature.

Vault is the largest nonicosahedral cytosolic nucleoprotein particle. In cancer cells, the upregulation of vault components, including 3 major vault proteins (major vault proteins [MVPs]) and 4 vault RNAs such as vtRNA1-1, vtRNA1-2, vtRNA1-3, and vtRNA2-1, is associated with the chemoresistance.[28] Together with its role on chemoresistance, Amort et al identified a role of the noncoding vtRNA1-1 on inhibiting both the extrinsic and the intrinsic apoptotic pathways through a nuclear factor (NF)-κB.[29] In primary tumor group of our study, upregulation of vtRNA1-1 was determined. Thinking of the metastasis capability of primary tumor cells to peritoneal implant, vtRNA1-1 upregulation can be facilitated metastasis process with the inhibition of apoptosis.

The melanoma cell adhesion molecule (MCAM; also known as MUC18 or CD146), a member of immunoglobulin superfamily, is a membrane calcium-independent glycoprotein adhesion molecule. The MCAM expression was observed in a variety of tumor tissues such as melanoma, breast cancer, and colorectal cancer, and this upregulation was associated with metastasis. Wu et al showed that MCAM-positivity rate was higher in metastatic ovarian cancer lesions compared to ovarian tumors and malignant epithelial ovarian cancer. Also, increased apoptosis was observed followed by MCAM silencing in the ovarian cancer cell line SKOV-3, which presents high MCAM positivity.[30] Our results were in concordance with Wu et al results, which show higher MCAM upregulation in peritoneal metastatic implants.

Although there were not many differences between primary tumor and peritoneal tumor implants in terms of gene expression, there were 979 genes that were differentially expressed between primary tumor and malignant cells in the ascites (Supplemental Table 2); additionally, 649 genes were differentially expressed between malignant cells in the ascites and peritoneal implant (Supplemental Table 3). Those differences mainly overlap with each other, and they had a lot of common diversities regarding gene expression depending on free malignant cells in the ascites.

USP17 gene family, *SNORD* and *SNORA* gene family, and *RPS* (*RPS3A*, *RPS2*, and *RPS8*) and *RPL* gene family members (*RPL13A* and *RPL23A*) were mainly differentially expressed genes between primary tumor and malignant cells in the ascites and between malignant cells in the ascites and peritoneal implants.

Furthermore, functional enrichment analysis was conducted to discriminate altered biological processes related to metastasis. Three common GO-enriched processes were protein deubiquitination (GO:0016579), ubiquitin-dependent protein catabolism process (GO:0006511), and apoptotic process (GO:0006915). All differentially expressed genes related to those processes belong to *USP17* (ubiquitin-specific protease 17) gene family (Table 2). Deubiquitinating (DUB) enzymes, which have approximately 100 subtypes, can be classified into 5 different groups: cysteine proteases ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph domain proteases, metalloproteases JAB1/MPN/MOV34 (JAMM), and USPs.[31] The USPs are the biggest group of DUBs, and in humans, USP family has approximately 56 members. The DUB/USP17 subfamily is cytokine-inducible cysteine proteases identified by the inclusion of typical histidine and cysteine boxes within their catalytic domain. In recent years, studies suggest that the DUB/USP17 family regulate specific processes such as NF-κB activation, the initiation of DNA repair, cell growth, survival, cell-cycle arrest, and apoptosis.[32-34] In our study, *USP17* gene family, such as DUB enzymes, were upregulated in malignant cells in the ascites when compared to primary tumor and peritoneal implant. So deubiquitinase inhibition can be seen as a chemotherapeutic target.

Ubiquitin is a protein found in eukaryotic cells and determines de/stabilization, localization, and activity of its target protein. Its ligation to target protein is denoted as "kiss of death," and mostly, target protein is marked for degradation in proteasomes. Additionally, it regulates many functions such as apoptosis, cell division and proliferation, immune response, organelle biogenesis, and receptor modulation.[35] Ubiquitin modifications of proteins are controlled by attachment (E1, E2, E3) and detachment enzymes. The latter was called deubiquitinating (DUB) enzymes and was classified into 6 groups: USPs, ubiquitin COOH-terminal hydrolases (UCH), OTUs, Josephins, JAMM family, and motif interacting with ubiquitin-containing novel DUB family (MINDY).[36] Recent

studies mentioned about the relationship between the somatic/germline mutations or changes in expression of genes coding DUB enzymes and human cancers. The DUB enzymes were found responsible in many functions of metastasis such as EMT, cell-fate regulation, anoikis resistance, and extracellular matrix degradation.[37] In a study, Xing et al reported that USP4 inhibited PLR3 (PTP4A3) degradation and intracellular accumulation of PRL-3 resulted in activation of AKT pathway and decrement in E-cadherin in colorectal cancer cells.[38] In another study, Hu et al concluded that *USP22* induced EMT and increased cell invasion in lung adenocarcinoma cells. Moreover, *USP22* upregulated *TGFB1* expression and was found to be independent prognostic factor for both overall and disease-free survival in lung cancer.[39]

There are few studies regarding ubiquitin/deubiquitination and ovarian cancer pathogenesis. Jensen et al identified Bap1 protein, which is a ligand of BRCA1 and acts as ubiquitin hydrolase. They reported that BAP1 might inhibit BRCA1-associated cell proliferation.[40] Similarly, Rao et al mentioned in a review that ubiquitination might interfere with *ERK*, *CDK,* and *p53* pathways and *ERBB2* gene expression in ovarian cancer.[41]

A relevant study with our research is the Wang et al's study regarding *USP14* and ovarian cancer. They found USP14 enzyme level significantly higher in patients with ovarian cancer compared to controls. In SKOV-3 cell lines, they reported that expression of *USP14* increased proliferation and downregulation of *USP14* induced apoptosis. They concluded that *USP14* might be a therapeutic target for ovarian cancer.[42]

Another process that the candidate genes were significantly differently expressed between the study groups was apoptotic process (GO:0006915). Burrows et al reported that USP17 (DUB3) enzyme was induced by interleukin-4 and interleukin-6 stimulation and might regulate immune function. Additionally, they demonstrated that expression of DUB3 blocked cell proliferation and initiated apoptosis.[43] In our study, *USP17* was overexpressed in the malignant cells in ascites compared to primary tumor and peritoneal implant. According to this, upregulation of *USP17* in free malignant cells can explain the apoptosis resistance in cells, which have metastasis capability. In Burrows et al's study, USP17 expression induced apoptosis; it can also be explained as downregulation of USP17 inhibits apoptosis. With this approach, it can be concluded that Burrows et al's study is in concordance with our study. Since the cell types in the latter study were other than ovarian cells, further studies performed in ovarian cancer cell lines are warranted to see the effects of USP17 on apoptosis.

In our study, we reported for the first time that genes belong to *USP17* family were significantly differently expressed between "primary tumor and free malignant cells in the ascites" and "peritoneal implant and free malignant cells in the ascites" in ovarian cancer. It was previously reported that *USP17* controlled cell-cycle progression by preventing the catabolism of *CDC25A* and had an important role in tumorigenesis in terms of cell proliferation and invasion.[32,44] There is no previous study regarding ovarian cancer and *USP17*, but few studies were reported with regard to non–small cell lung cancer (NSCLC) and osteosarcoma (OS) and *USP17.* In a previous study, McFarlane et al reported that USP17-positive patients with NSCLC had significantly reduced recurrence-free survival and *USP17* levels may correlate with NSCLC distant metastasis.[45] Similarly, Zhang et al mentioned that *USP17* silencing downregulated the expression of matrix metalloproteases (MMP3 and MMP9) in NSCLC cells. Furthermore, animal model results showed that USP17 suppression inhibited NSCLC tumorigenesis and growth.[46] In a very recent study, Song et al reported that *USP17* was upregulated in OS tissues and cell lines. The USP17 promoted EMT by cancer cell proliferation, migration, and invasion. Furthermore, USP17 also stabilized SMAD4 through its DUB activity.[47] Under all of the results from these studies, it can be said that upregulation of USP17 facilitates the metastatic processes by inducing invasion, EMT, and proliferation by enhancing DUB. Our study presents upregulation of *USP17* gene family members in free ovarian cancer cells in the ascites. Once cancer cells reach the circulation, most cells tend to die, so they undergo apoptosis due to the lack of cellular attachments, components of immune system, shear stress, or oxidative stress. To avoid immune surveillance, cancer cells express different surface proteins for the formation of clusters with different cells in circulation and enhance their camouflage capacity to escape apoptosis. They also avoid oxidative stress by reorganizing their metabolism and at the end they develop resistance to apoptosis. At this process, cancer cells reorganize their metabolism by changing the expression levels of key molecules, such as surface proteins and apoptosis-related proteins. In our study, we showed that EMT exists with a phenotypic switch from epithelial to mesenchymal phenotype in the free ovarian cancer cells in the ascites and also that 3 common GO-enriched processes such as protein deubiquitination (GO:0016579), ubiquitin-dependent protein catabolism process (GO:0006511), and apoptotic process (GO:0006915) appeared due to the upregulation of *USP17* gene family members such as DUB enzymes.

The other differentially expressed genes related to significantly enriched GO functions and KEGG pathways were related to ribosomal proteins and ribosome pathway. One of them, RPS2, was previously reported to be associated with colon and prostate cancer.[48] They demonstrated that knockdown of *RPS2* expression inhibited cell growth and induced apoptosis in the malignant prostate cells. MDM2-p53 feedback loop is regulated by ribosomal protein-mediated p53 activity, such as RPL4, RPS14, and RPS7. Additionally, RPL34 could mediate the upstream factors (FOXO families) or downstream proteins (mTOR and AMPK) of p53 signaling pathway in pancreatic cancers.[49] A similar gene, *RPL22L1*, which was not significantly differentially expressed in our study, was reported to upregulate the mesenchymal markers VIM, fibronectin, and α-SMA and reduce the expression of epithelial markers CDH1, CTNNA1, and CTNNB1 in ovarian cancer cell lines.[50] The role of ribosomal proteins in EMT of ovarian cancer cells should be further investigated.

This is the first study that shows a perspective on differentially expressed genes in EMT of high-grade serous ovarian cancer. Further studies should focus on confirming the impact of candidate genes found in this study in order to define new biomarkers or targeted therapeutic agents in ovarian cancer. Taken as a whole, the role of ribosome biogenesis in the EMT process is evident, and there is remarkable evidence that the process is ubiquitin–proteasome dependent. Additionally, the role and molecular mechanism of *USP17* in carcinogenesis and metastasis of ovarian cancer should be investigated in both in vitro studies and large volume clinical studies.

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Supplemental Material

Supplemental material for this article is available online.

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