

Circulating Tumor Cell-Free DNA Genes as Prognostic Gene Signature for Platinum Resistant Ovarian Cancer Diagnosis

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ABSTRACT: Clinical management of gynecological cancer begins by optimal debulking with first-line platinum-based chemotherapy. However, in ~80% patients, ovarian cancer will recur and is lethal. Prognostic gene signature panel identifying platinum-resistance enables better patient stratification for precision therapy. Retrospectively collected serum from 11 “poor” (<6 months progression free interval [PFI]) and 22 “favorable” (>24 months PFI) prognosis patients, were evaluated using circulating cell-free DNA (cfDNA). DNA from both groups showed 50 to 10 000 bp fragments. Pairwise analysis of sequenced cfDNA from patients showed that gene dosages were higher for 29 genes and lower for 64 genes in poor than favorable prognosis patients. Gene ontology analysis of higher dose genes predominantly grouped into cytoskeletal proteins, while lower dose genes, as hydrolases and receptors. Higher dosage genes searched for cancer-relatedness in Reactome database indicated 15 genes were referenced with cancer. Among them 3 genes, TGFBR2, ZMIZ2, and NRG2, were interacting with more than 4 cancer-associated genes. Protein expression analysis of tumor samples indicated that TGFBR2 was downregulated and ZMIZ2 was upregulated in poor prognosis patients. Our results indicate that the cfDNA gene dosage combined with protein expression in tumor samples can serve as gene signature panel for prognosis determination amongst ovarian cancer patients.

KEYWORDS: Biomarker, cfDNA, TGFBR2, ZMIZ2, ovarian cancer

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Introduction

Cancer heterogeneity exists both functionally and morphologically, amplifying the challenges of effectively treating the disease.¹ Therapy stratification tailored to suit the precise characteristics of each patient yields a better prognosis.² Biopsies with specific indicative property, also known as biomarkers, can be expression levels of proteins or RNA, copy number variation of specific genes, micro-RNA, or non-coding RNA.³ Genomic, proteomic, and other “-omic” approaches to evaluate tumor samples have aided in developing gene panels of predictive biomarkers, which identify patients who will respond to distinct therapy strategies.⁴ In order to minimize invasiveness in generating samples, liquid biopsies from body fluids such as blood, saliva, and urine have been investigated to develop biomarker gene panels.⁵

For nearly 70 years, we have known that there are circulating nucleic acids within the blood,⁶ but the concept gained importance in the late 1970s when serum of cancer patients was noted to have a higher concentration of nucleic acids compared to healthy individuals.⁷ Hypothesizing that circulating tumor cell-free DNA (cfDNA) can provide potential

clues related to primary and metastatic tumors, studies on these DNA have been pursued in many cancer types.⁸ Ovarian cancer is the most lethal of all gynecologic malignancies. For 2021, it is estimated that there would be 21 410 new cases and 13 770 deaths.⁹ Despite response to first-line therapy, ~80% of patients will experience disease recurrence, and unfortunately, emergence of resistance to chemotherapy is inevitable for most patients.^{10–12} Platinum-based chemotherapy is the single most active treatment strategy for ovarian cancer, and the underlying mechanisms of resistance to platinum-based chemotherapy are most likely multifactorial.^{13–15} These include neutralizing alterations by cancer cells to platinum-based intercalated DNA adducts and epigenetic responses of the chemotherapy.¹⁶ Hence, resistance to platinum-based chemotherapy is a major challenge for clinicians, with median progression free survival of 4 months, and median overall survival of 12 months for these patients.^{17,18} Development of a gene signature panel for reliable prediction of prognosis prior to manifestation of chemo-resistance may help risk-stratify patients with more aggressive disease for whom treatment approaches could be altered.



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Although cancer patients have higher concentration of cfDNA than healthy individuals,^{7,19–21} the concentration and gene composition of cfDNA vary amongst cancer patients.²² Prior research has shown that DNA encoding oncogenes, tumor suppressor genes, microsatellite alterations, and hypermethylated genes are circulating in the plasma of cancer patients.²³ cfDNA levels in the serum has been shown to predict response to therapy,²⁴ and qualitative evaluation of cfDNA methylation has been used to differentiate cancer survival.^{25,26} However, the development of precise gene signature panels with specific genes in the serum is incomplete. Rapid and accurate methods of determination may provide better prediction of response to anti-cancer therapies. Based on this rationale, we sought to develop a precise prognostic gene signature panel of cfDNA genes in poor prognosis (platinum free interval [PFI] < 6 months) versus favorable prognosis (PFI > 24 months) patients with high grade serous ovarian cancer.

Our results on sequencing the cfDNA isolated from poor and favorable prognosis patient serum samples indicated that a distinct cluster of genes as DNA fragments are circulating in the blood. Based on the quantification of these genes, they were grouped as genes of higher or lower dosage in the poor prognosis patients compared to favorable prognosis patients. Those high dose genes observed in poor prognosis patient samples were compared to the Reactome database for cancer relevance. Transforming growth factor, beta receptor II (TGFBR2) and Zinc finger, MIZ-type containing 2 (ZMIZ2), which indicated multiple interactions with cancer associated proteins, were verified for protein expression in patient tumor tissues. Based on our study, we propose a novel multi-factorial approach to develop a gene signature panel, with genes identified in cfDNA fragments of patient serum and their protein expression levels in tumor tissues that could demarcate prognosis in ovarian cancer patients.

Materials and Methods

Patient selection

Eligibility was restricted to women with stage IIIC high grade serous carcinoma of the ovary, fallopian tube, or peritoneum who underwent primary debulking surgery followed by adjuvant platinum-based chemotherapy. All women were treated by gynecologic oncologists at Stephenson Cancer Center at The University of Oklahoma Health Sciences Center. In order to compare groups with distinct prognosis and clinical course, patients were dichotomized into 2 groups based on their time from completion of platinum-based chemotherapy until first recurrence (PFI). PFI < 6 months was selected as the poor prognostic group, and PFI ≥ 24 months defined the favorable prognostic cohort. The study methods and experimental protocols were approved by the University of Oklahoma Health Science Center, Office of Human Research Participant Protection, Institutional Review Board (IRB approval # 6404). The authors confirm that the methods were carried out in

accordance with relevant guidelines and regulations. All patients in the study were above 18 years and were informed of the nature of the study and provided written consent, prior to treatment and collection of samples. The storage of samples was done by Stephenson Cancer Center Biospecimen core. Deidentified serum and tissue samples were made available to the researchers for this study. TMA of 126 ovarian cancer patients and pertaining deidentified patient characteristics were graciously provided by the authors of earlier publication.²⁷

cfDNA isolation

CfDNA from the 2 ml of retrospectively collected serum from each patient was isolated using *Quick-cfDNA*TM Serum & Plasma Kit (Zymo Research) following the manufacturer's protocol. The DNA was eluted in 25 µl of elution buffer and the quantification was done using Qubit kit on a Qubit fluorometer (Thermo Fisher). An estimated 1 ng/µl of sample were loaded on High Sensitivity DNA chip (Agilent) and run on a Bioanalyzer 2100 (Agilent). The DNA integrity and purity were evaluated. The electrographs were generated using the 2100 expert software (Agilent).

Sequencing and analysis of cfDNA

For sequencing of the cfDNA, a total of 1 ng of cfDNA was used to construct library using the Nextera XT DNA library preparation kit (Illumina). The samples were sequenced on MiSeq system (Illumina) at 2 × 250 base pair read length. The sequencing yielded about 60 million reads and the quality averages were ≥ Q30 in 71% or more reads. The sequences were aligned against the human genome sequence (GRCh build 38) assembled by Genome Reference Consortium and a pairwise sequence data analysis was performed using GeneSifter analysis software (Perkin Elmer). Annotated genes were normalized to their reads per kilobase per million mapped reads (RPKM) for each sample and gene count were averaged for each group of patients. Gene copy number variations were statistically evaluated following the Welch's *t*-test method with *P* < .05. Volcano plot for the genes that showed fold change of > 1 and < -1 in poor compared to favorable prognosis patient groups was performed. Heatmap for the gene copy number variation and the ratio of poor prognosis patient genes, grouped either higher (≥ 1.5-fold) or lower (≤ -1.5-fold) compared to favorable prognosis patient genes, were generated using ggplot2 algorithm of the R program.

Gene ontology analysis

Both groups, high and low dose genes, were analyzed using the PANTHER (protein annotation through evolutionary relationship) system for their gene ontology.²⁸ Classification of these genes into various groups was based on their biological processes, protein class, or molecular functions. This was

Table 1. Clinical characteristics of patients selected in the study.

CHARACTERISTICS	FAVORABLE PROGNOSIS	POOR PROGNOSIS	P-VALUE
Age at diagnosis	58.4 ± 2.3	67 ± 3.8	.0355
Platinum free interval	47.4 ± 5.8	2 ± 0.7	.0001
Body mass index	24.14 ± 1.4	26.07 ± 2.0	.2222
Initial CA125 (units/ml)	1568.1 ± 469.6	1824.2 ± 799.3	.3931
cfDNA conc (ng/μl)	1.53 ± 0.5	1.51 ± 0.6	.8692

performed using PANTHER on their website, www.pantherdb.org.

Immunohistochemical staining of tissue microarray (TMA)

All the concomitant patient FFPE samples were collected. Hematoxylin-Eosin (HE) stained sections were marked for tumor by a pathologist. Triplicate tumor cores of 1 mm diameter were set in an empty block using Tissue Arrayer ATA110C (Veridiam) to develop a TMA. For control, normal uterus and fallopian tube were used. The TMA was constructed at the Stephenson Cancer Center's Tissue Pathology Shared Resource using standard methodology.²⁹ TMA of 126 ovarian cancer patient tumor samples were provided by the authors of an earlier publication.²⁷ IHC-staining of both TMA with the antibodies of interest was carried out at the SCC Tissue Pathology Shared Resource that utilizes automated Leica Bond III for IHC staining. Antibodies used were TGFBR2 (1:400; BLOSS) and ZMIZ2 (1:30; Novus Biologicals). IHC staining method were followed as described in publication,³⁰ briefly, FFPE tissues were sectioned. Deparaffinization and rehydration were performed on an automated Multistainer (Leica ST5020). Subsequently, these slides were transferred to the Leica Bond III™, where target retrieval and peroxidase-blocking were performed. It was followed by the primary antibody incubation for 60 minutes and Poly-HRP IgG reagent was used as secondary antibody. DAB (3,3'-diaminobenzidine tetrahydrochloride) was used as chromogen and slides were counterstained with hematoxylin. Stained slides were dehydrated (Leica ST5020) and mounted (Leica MM24). Antibody specific positive control and negative control (omission of primary antibody) were parallel-stained.

Scanning and analysis of IHC slides

Methods for scanning and analysis of the IHC stained slides have been adapted similar to the earlier publication.³⁰ Briefly, the IHC stained TMA slides were scanned using an Aperio slide scanner (Leica Inc.). The TMA were segmented, and individual spots were assigned to respective patients using the TMA

lab software, which works in conjunction with the spectrum software from Leica Aperio. Quantification of the various protein staining was done using Spectrum software (Leica Inc.).

Software used

Cytoscape 3.4v with plugin for Reactome FIViz app 6 with FI network style for connections. R program with ggplot2 package using R-Studio was used to generate the heatmap for the genes. Multiple unpaired *t*-tests were done for patient analysis and for individual protein expression studies *t*-tests were done for all the TMA analysis. All statistical analysis was done using Graphpad Prism 7.0v.

Results

Two cohorts of retrospective ovarian cancer patient samples were used in this study. The primary cohort for the cfDNA analysis included 33 patients with either <6-month (poor prognosis; 11 patients) or ≥24-month (favorable prognosis; 22 patients) PFI. In order to maintain clinico-pathological uniformity, only patients with stage IIIC high grade serous ovarian cancer who underwent primary debulking surgery were included in the study. The majority of patients were Caucasian with similar BMI and initial serum CA125 concentration (units/milliliter). On average, women diagnosed at younger age (61.2 ± 1.7 year) favored longer (≥24 months) survival (Table 1). A secondary cohort included 126 ovarian cancer patients and the tumor samples from these patients represented in a TMA²⁷ were analyzed for validation of the results. Adapting the above criteria, this cohort was divided into 3 categories, poor prognosis (26 patients), favorable prognosis (32 patients), and rest of the patients between 6 and 24 months PFI (68 patients). The characteristics of the poor and favorable prognosis patients had similar BMI, initial CA125 concentration, and age of diagnosis (Supplemental Table 1).

cfDNA characteristics and sequence analysis

Retrospective serum collected at the time of diagnosis from first cohort of both poor and favorable prognosis patients were

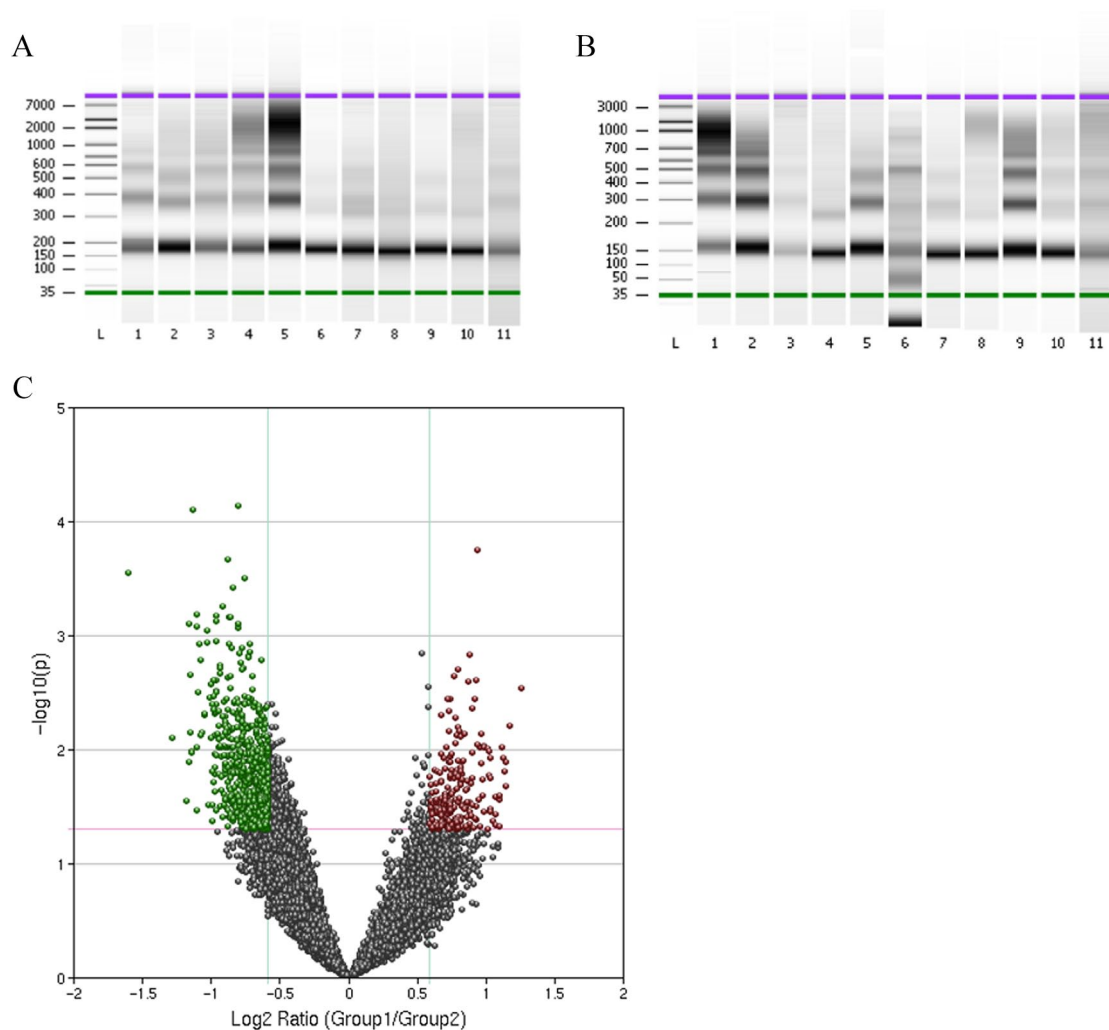


Figure 1. Identification of higher and lower dose of genes in the cfDNA isolated from serum of platinum resistant (poor prognosis) and sensitive (favorable prognosis) patients. (A and B) Chromatogram generated for cfDNA isolated from serum of patients using High sensitivity DNA analysis chip. (A) Favorable and (B) Poor prognosis. (C) Volcanic plot showing genes of higher and lower dose in sequenced cfDNA isolated from patients with poor and favorable prognosis. *P*-value cutoff: 0.05 (red line); lower threshold: 1.5 (green line); higher (red spheres); and lower (green spheres) genes.

used to extract the cfDNA. Concentration of cfDNA in the serum did not vary among both groups of patients (Table 1). The molecular size of cfDNA fragments obtained in the serum were between 50 and 10000 base pairs (Figure 1A and B).

An unbiased next generation sequencing to delineate the gene composition in the cfDNA of the test samples was carried out following previously published procedures.^{31,32} The cfDNA fragments were sequenced and annotated against the human genome sequences (GRch build 38). Copy number variation of the cfDNA for each gene for each group of patients was calculated. Comparing the gene copy number variations between the poor and favorable prognosis patients, gene dosage for each group of patients was calculated. A volcano plot was generated following previously published methods.³³ The volcano plot from the results representing the gene dosage of >1 and < -1 for the poor prognosis compared to the favorable prognosis patients was constructed and presented in Figure 1C. In order to avoid losing physiologically critical

genes and signaling pathway proteins that may play a crucial role, an arbitrary gene dosage cut-off of 1.5-fold variation was chosen based on previous studies.^{34,35} Using a cutoff of 1.5-fold gene dosage and <0.01 *P*-value threshold, 29 genes were identified to be of higher and 64 genes of lower gene dosages in the poor prognosis compared to favorable prognosis patients (Tables 2 and 3).

Gene ontology analysis on genes

Gene ontology enrichment analysis was performed using the PANTHER classification system.²⁸ A heatmap for the copy number variation and ratio of the higher dose genes in the poor and favorable prognosis patients were plotted (Figure 2A; Table 2). Similarly, a heatmap of the copy number variation was plotted for the 69 lower dosage genes that had 1.5-fold or lower fold ratio in the poor prognosis compared to favorable prognosis patients (Figure 3A; Table 3).

Table 2. cfDNA higher dosage genes in the poor prognosis ovarian cancer patients.

GENE	GENE NAME	FAVORABLE	POOR	P-VALUE
MVP	Major vault protein	4.14	7.89	.0002
C16orf53	Chromosome 16 open reading frame 53	2.51	4.61	.0014
PLEC1	Plectin 1, intermediate filament binding protein 500 kDa	7.79	13.50	.0020
DLGAP2	Disks, large (Drosophila) homolog-associated protein 2	4.36	7.41	.0022
KRT9	Keratin 9	1.41	2.67	.0024
KDM5C	Lysine (K)-specific demethylase 5C	4.42	8.08	.0025
MGAT4B	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B	1.61	3.84	.0029
ZMIZ2	Zinc finger, MIZ-type containing 2	3.43	5.65	.0035
C17orf68	Chromosome 17 open reading frame 68	3.83	7.25	.0036
GPR176	G protein-coupled receptor 176	2.48	4.15	.0036
KIF17	Kinesin family member 17	2.71	5.06	.0043
ADAMTSL3	ADAMTS-like 3	3.96	6.58	.0045
UNC5B	Unc-5 homolog B (<i>C. elegans</i>)	2.82	4.83	.0052
CORO6	Coronin 6	1.63	3.67	.0062
PANX2	Pannexin 2	1.63	2.83	.0063
DKFZp686O24166	Hypothetical protein DKFZp686O24166	2.88	4.77	.0068
HIP1R	Huntingtin interacting protein 1 related	2.86	4.99	.0069
QRICH2	Glutamine rich 2	3.78	7.37	.0072
NRG2	Neuregulin 2	1.84	3.29	.0073
COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)	2.54	4.38	.0074
CA12	Carbonic anhydrase XII	2.15	3.78	.0076
MYO1H	Myosin IH	2.49	4.27	.0088
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	1.97	3.77	.0090
TGFBR2	Transforming growth factor, beta receptor II (70/80 kDa)	1.98	3.90	.0091
LOC653781	Similar to protein expressed in prostate, ovary, testis, and placenta 2	1.59	3.15	.0093
PKP1	Plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	3.95	6.47	.0094
DGCR14	DiGeorge syndrome critical region gene 14	2.16	4.70	.0094
C1orf2	Chromosome 1 open reading frame 2	1.79	3.47	.0094
SLC10A3	Solute carrier family 10 (sodium/bile acid cotransporter family), member 3	1.44	2.91	.0098

Table 3. cfDNA lower dosage genes in the poor prognosis ovarian cancer patients.

GENE	GENE NAME	FAVORABLE	POOR	P-VALUE
OSBP	Oxysterol binding protein	2.88	1.32	.0001
ARFGAP2	ADP-ribosylation factor GTPase activating protein 2	3.00	1.63	.0002
FAM105A	Family with sequence similarity 105, member A	3.39	1.11	.0003
ZNF652	Zinc finger protein 652	3.52	1.81	.0007

(Continued)

Table 3. (Continued)

GENE	GENE NAME	FAVORABLE	POOR	P-VALUE
CTNNA2	Catenin (cadherin-associated protein), alpha 2	2.44	1.09	.0008
C6	Complement component 6	2.49	1.16	.0008
RAGE	Renal tumor antigen	2.08	1.02	.0009
TBC1D8B	TBC1 domain family, member 8B (with GRAM domain)	2.19	1.12	.0011
CCDC120	Coiled-coil domain containing 120	3.14	1.53	.0011
UBE2L3	Ubiquitin-conjugating enzyme E2L 3	2.57	1.21	.0012
LOC100130698	Hypothetical LOC100130698	4.71	2.24	.0016
PBX1	Pre-B-cell leukemia homeobox 1	2.99	1.56	.0021
GLUD2	Glutamate dehydrogenase 2	2.60	1.17	.0022
STIM1	Stromal interaction molecule 1	3.33	1.70	.0024
DGKK	Diacylglycerol kinase, kappa	4.50	2.28	.0025
DNAL1	Dynein, axonemal, light chain 1	2.79	1.40	.0027
POU2F3	POU class 2 homeobox 3	2.52	1.17	.0031
ALG13	Asparagine-linked glycosylation 13 homolog (<i>S. cerevisiae</i>)	2.99	1.53	.0032
FOSL2	FOS-like antigen 2	3.78	2.14	.0036
FKBP9L	FK506 binding protein 9-like	2.15	1.21	.0038
TM4SF18	Transmembrane 4I six family member 18	2.53	1.35	.0038
POLQ	Polymerase (DNA directed), theta	2.93	1.48	.0044
CD163L1	CD163 molecule-like 1	3.37	1.82	.0045
FAM55C	Family with sequence similarity 55, member C	3.94	2.04	.0045
FAM123B	Family with sequence similarity 123B	7.65	4.27	.0047
GPR119	G protein-coupled receptor 119	2.61	1.33	.0047
IDE	Insulin-degrading enzyme	2.76	1.41	.0048
SLC22A17	Solute carrier family 22, member 17	2.34	1.13	.0048
CNTLN	Centlein, centrosomal protein	3.63	1.75	.0050
ZNF235	Zinc finger protein 235	2.90	1.67	.0054
GPR112	G protein-coupled receptor 112	7.12	4.15	.0056
EAF1	ELL associated factor 1	3.01	1.62	.0056
TRIM13	Tripartite motif-containing 13	2.51	1.36	.0061
NPY2R	Neuropeptide Y receptor Y2	2.60	1.40	.0061
RRP8	Ribosomal RNA processing 8, methyltransferase, homolog (yeast)	2.73	1.42	.0061
PCYT1B	Phosphate cytidyltransferase 1, choline, beta	4.02	2.16	.0062
CRYBG3	Beta-gamma crystallin domain containing 3	5.00	2.85	.0062
CD207	CD207 molecule, langerin	2.39	1.34	.0065
XRN1	5-3 exoribonuclease 1	2.40	1.51	.0068
ZNF208	Zinc finger protein 208	4.46	2.40	.0069

(Continued)

Table 3. (Continued)

GENE	GENE NAME	FAVORABLE	POOR	P-VALUE
KIAA1919	KIAA1919	2.48	1.18	.0070
SLC22A4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	2.12	1.15	.0070
MCART6	Mitochondrial carrier triple repeat 6	3.09	1.39	.0070
SSR1	Signal sequence receptor, alpha	3.30	1.94	.0070
PREX2	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	2.54	1.31	.0072
MYH16	Myosin, heavy chain 16 pseudogene	2.82	1.44	.0074
LRRRC33	Leucine rich repeat containing 33	2.76	1.31	.0074
CD96	CD96 molecule	2.43	1.28	.0076
PCDH15	Protocadherin 15	4.24	2.12	.0079
ALG11	Asparagine-linked glycosylation 11, alpha-1,2-mannosyltransferase homolog (yeast)	2.50	1.42	.0083
TREX1	Three prime repair exonuclease 1	2.50	1.46	.0083
DOK6	Docking protein 6	3.81	2.28	.0089
ITPR1	Inositol 1 and 4,5-triphosphate receptor, type 1	6.77	4.43	.0091
ZDHHC22	Zinc finger, DHHC-type containing 22	3.18	1.69	.0091
TFAP2E	Transcription factor AP-2 epsilon (activating enhancer binding protein 2 epsilon)	2.73	1.56	.0093
NCBP1	Nuclear cap binding protein subunit 1 and 80kDa	2.58	1.35	.0093
CASC5	Cancer susceptibility candidate 5	2.35	1.42	.0094
MPL	Myeloproliferative leukemia virus oncogene	2.79	1.74	.0094
LRRRC66	Leucine rich repeat containing 66	2.55	1.65	.0095
LSM8	LSM8 homolog, U6 small nuclear RNA associated (<i>S. cerevisiae</i>)	3.59	1.99	.0095
NONO	Non-POU domain containing, octamer-binding	2.11	1.11	.0096
NDRG3	NDRG family member 3	2.50	1.29	.0096
ANKRD54	Ankyrin repeat domain 54	2.90	1.35	.0096
KCNJ16	Potassium inwardly-rectifying channel, subfamily J, member 16	2.32	1.43	.0100

Higher dose genes. The 29 genes identified as higher dose genes in poor outcome patients were subjected to gene ontology grouping. Based on the biological processes genes showed clustering toward cellular processes (29%), developmental processes (14%), and cellular biogenesis (12%) (Figure 2B). Many of these genes were classified as cytoskeletal proteins (23%), and about 9% each as enzyme modulators, transcription factors, nucleic acid binding proteins, receptors, and signaling proteins (Figure 2C). Furthermore, these factors reflect in their molecular function classification, attributing them to protein binding function (47%) and catalytic activity (27%) (Figure 2D).

Lower dose genes. Gene ontology analysis showed that these lower dose genes were involved in cellular processes (36%), metabolic processes (18%), stimulus response (13%), and

biological regulations (11%) of the cell (Figure 3B). Distinctly different from higher dosage genes, protein classification showed a higher number of them as hydrolases (16%), receptors (16%), and nucleic acid binding proteins (14%) (Figure 3C). Agreeing with the biological process grouping, the molecular function classification of these genes indicated a greater number of lower dosage genes grouped with protein binding (40%), catalytic (21%), or receptor activity (16%) (Figure 3D).

Interaction nodes analysis of the higher dosage genes

Reactome database was searched using Cytoscape functional interaction network analysis module for cancer linked genes, to determine which of the higher dose genes formed nodes of interaction with cancer associated genes.³⁶ Figure 4 shows the degree-based circular layout representation of the analysis.

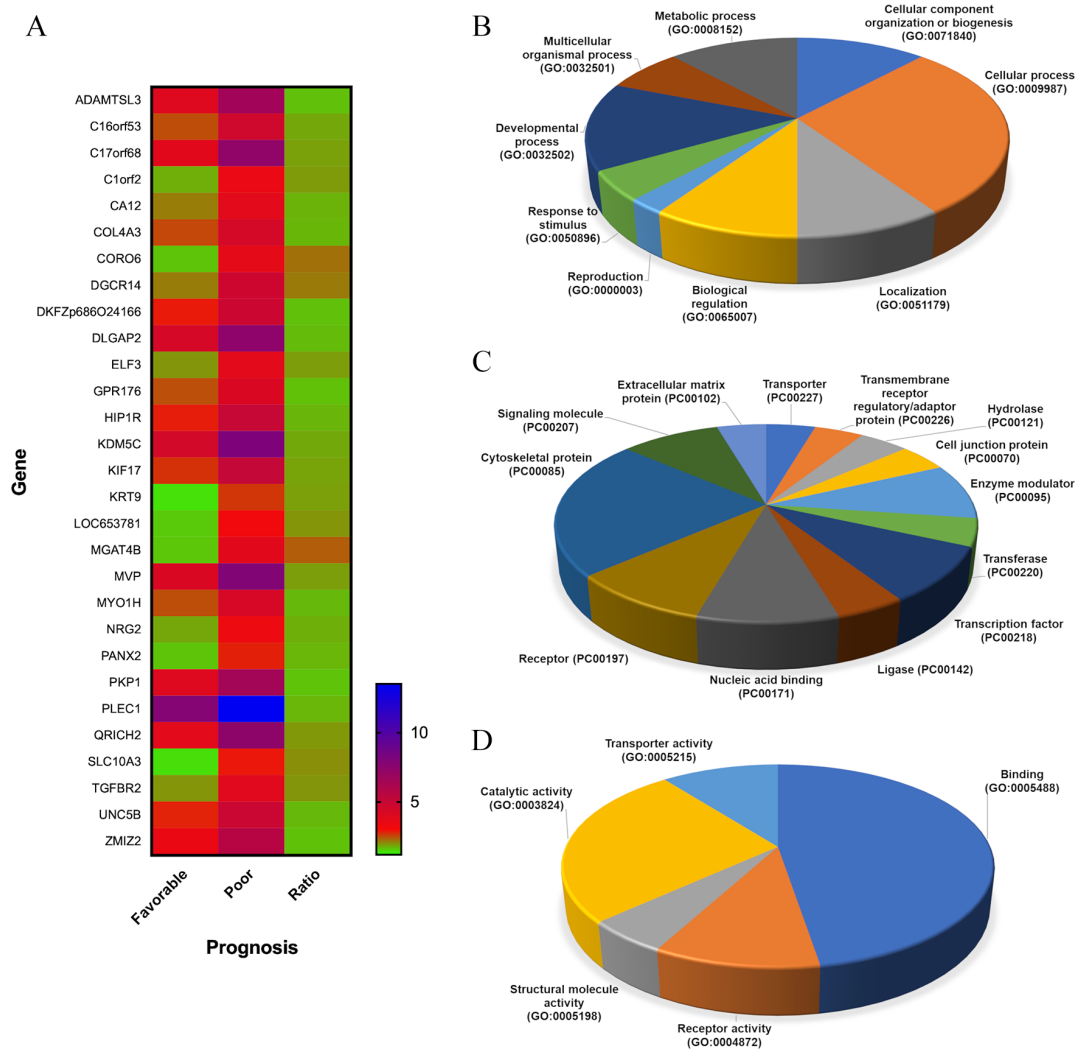


Figure 2. Ontology classification of higher dosage genes in poor compared to favorable prognosis patients into various relationship groups. (A) Heatmap representing absolute expression levels of higher dosage genes in corresponding poor and favorable prognosis patient serum. (B) Grouping of genes by their involvement in various biological processes, (C) classification of the genes based on their cellular functions, and (D) molecular functional grouping based on the molecular activities of their gene products.

Polyubiquitin-C precursor (UBC) is one of the linker genes used in the network analysis that showed 17 nodes, which had the highest number of nodes amongst all proteins (Supplemental Table 2). However, among the cfDNA higher dose genes, Transforming growth factor, beta receptor II (TGFBR2) was the hub for highest number of 5 nodes (Supplemental Table 2). Other major proteins that formed at least 2 node hubs were Zinc finger, MIZ-type containing 2 (ZMIZ2), Neuregulin 2 (NRG2), E74-like factor 3 (ets domain transcription factor) (ELF3), Unc-5 homolog B (UNC5B), and Huntingtin interacting protein 1 related (HIP1R) (Supplemental Table 2).

Validation of interaction network hub proteins of cfDNA in patient tumor samples

The higher dose genes, TGFBR2 and ZMIZ2, identified may be a consequence of gene amplification, which can lead to upregulation of the protein in the cancer cells. Hence,

immunohistochemistry (IHC) staining of TGFBR2 (Figure 5A, i) and ZMIZ2 (Figure 5B, i) were carried out on FFPE sections of the TMA constructed from ovarian cancer patient tumor samples. Since normal ovarian tissue was not available at that time of analysis, normal uterus and fallopian tube tissues were used as controls. The cell membrane receptor, TGFBR2 membranous expression IHC analysis showed increased expression in favorable patients compared to poor prognosis patients (Figure 5A, ii). IHC analysis of ovarian cancer patients for ZMIZ2 showed increased nuclear expression in poor patients compared to the favorable prognosis group of ovarian cancer patients (Figure 5B, ii). The expression levels of TGFBR2 and ZMIZ2 in the second cohort of 126 ovarian cancer patient tumor TMA was analyzed by IHC staining for the proteins. IHC analysis of TGFBR2 membranous expression level decreased in poor prognosis (Supplemental Figure 1A), while ZMIZ2 expression level increased in poor prognosis patients (Supplemental Figure 1B).

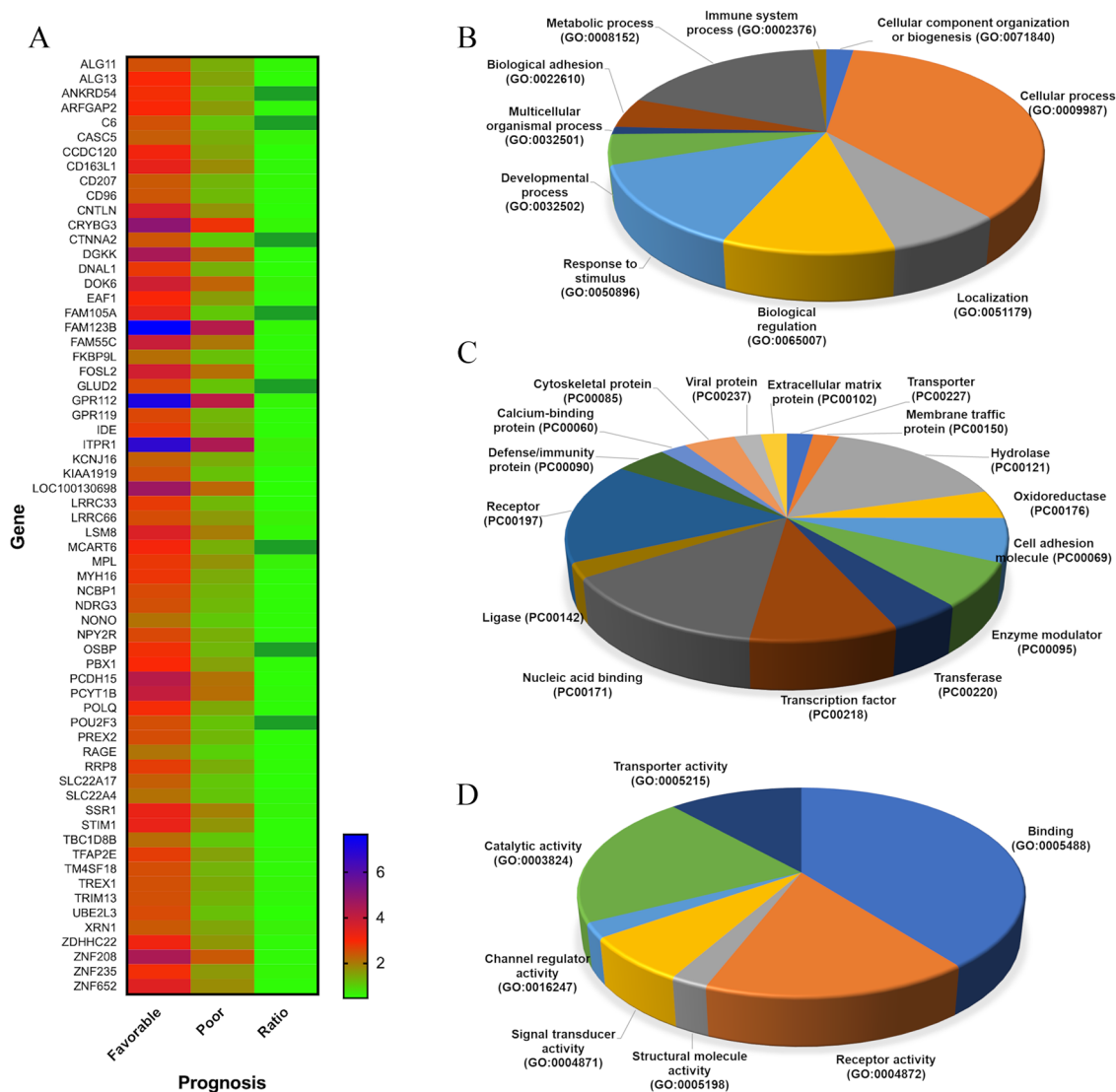


Figure 3. Ontology classification of lower dosage genes in poor compared to favorable prognosis patients into various relationship groups. (A) Heatmap representing absolute expression levels of lower dosage genes in corresponding poor and favorable prognosis patient serum. (B) Grouping of genes by their involvement in various biological processes, (C) classification of the genes based on their cellular functions, and (D) molecular functional grouping based on the molecular activities of their gene products.

Discussion

Resistance to platinum-based therapy is a major clinical challenge in caring for women with ovarian cancer.³⁷ Clinicians lack tools to preemptively identify platinum resistance, which cripples their effort to risk-stratify treatment options for ovarian cancer patients.³⁸ An ideal tool would be minimally invasive and rapidly testable method with high fidelity. Toward this goal, circulating tumor cell-free DNA (cfDNA) in the retrospective serum of poor and favorable prognosis patients, which were collected at the time of diagnosis, was analyzed herein. Hence, identification of prognostic gene signature that differentiate poor (PFI < 6 months) versus favorable (PFI > 24 months) prognosis patients, dichotomized based-on the time to their first recurrence, was analyzed in this study.

Previous studies have shown that the cfDNA in the serum has multiple origins including apoptotic or necrotic tumor

cells, exosomes, macrophage activity, and release by normal cells.³⁹⁻⁴³ The differential profile of genetic signatures in cfDNA could be the result of apoptotic cancer cell derived DNA,⁴⁴ chromosomal gene amplification/deletion,^{42,45} circulating tumor cell derived DNA,⁴⁶ or DNA specifically released as intratumoral signaling conduits by the cancer cells.^{47,48} Nevertheless, it can be posited that the cfDNA profile from poor versus favorable prognosis patients will exhibit variation in their respective gene composition. Hence, understanding the gene composition based on the cfDNA sequences would shed more light on the behavior of those cells that release the DNA.

Our sequence analyses of the cfDNA indicated that the samples from poor-prognosis patients exhibited 29 genes at higher dosage and 64 genes at lower dosage levels (Figure 1C; Tables 2 and 3). Many of the genes in the higher dosage group clustered toward functions essential in cancer cell invasion and

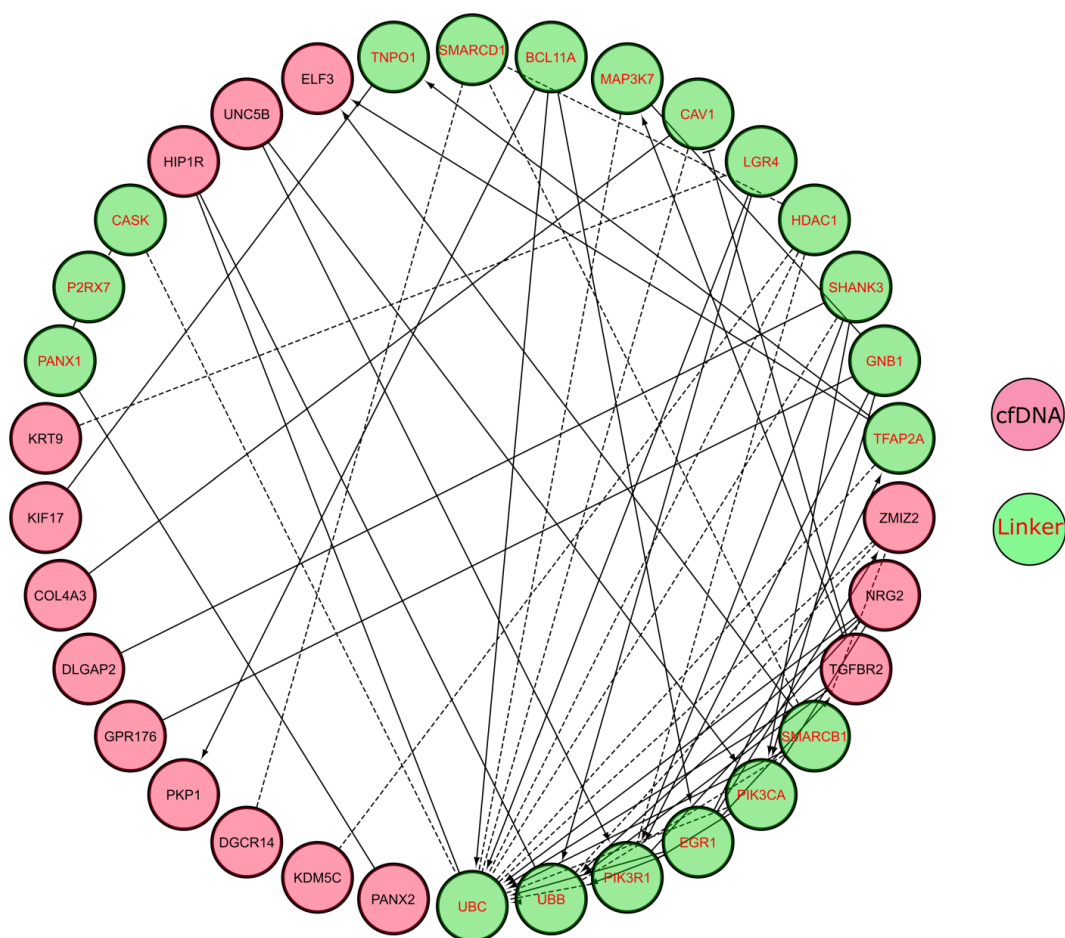


Figure 4. Identification of network gene nodes related to cancer among the higher dosage genes of poor compared to favorable prognosis patients. Interaction network nodes of higher dosage genes in circular layout generated using Reactome functional interaction network database on Cytoscape. Higher dosage genes (purple circles with black fonts) and linker genes (green circles with red fonts); solid black lines indicate known interactions and broken black lines are predicted interactions.

metastasis whereas the gene signature from the lower group consists mostly of genes encoding receptors and enzymes involved in growth inhibition or tumor suppression. Reasoning that the presence of specific gene signature rather than the lack of it or decrease in gene dosage levels could present a more reliable parameter for poor-prognosis, we focused on characterizing the gene signature associated with higher dosage genes.

All the 29 higher dosage genes from poor prognosis patients were investigated for cancer association. This search yielded at least 15 genes that were referenced to having functional relationship with some type of cancer. Specifically, increased expressions of Transforming growth factor beta receptor II (TGFBR2), Neuregulin 2 (NRG2) as well as the respective signaling pathways activated by the proteins in ovarian cancer progression and metastasis are well characterized.⁴⁹⁻⁵¹ TGFBR2 functional effect is impaired by YAP and EZH2 signaling in small cell lung cancer⁵² and by another protein, GFT2IRD1, in colorectal cancer.⁵³ NRG2 ligand binds with ErbB2 receptors and downstream signaling lead to colorectal cancer and breast cancer progression.^{54,55} Potential role of Keratin 5 (KRT5) as well as Collagen, type IV, alpha 3 (COL4A3) in invasive

metastasis and disease recurrence in ovarian cancer have also been previously known.^{56,57} Although a defined role for PANX1 in ovarian cancer is thus far unknown, it is shown to promote invasive migration in hepatocellular carcinoma.⁵⁸ Similarly, PKP1 has been shown to be associated with tumor progression and metastasis in prostate cancer and melanoma^{59,60} while Kinesin superfamily protein 17 (KIF17) is known to be associated with Rho-mediated epithelial cell migration.⁶¹ In addition, the poor-prognosis gene signature identified by our study also includes Zinc finger, MIZ-type containing 2 (ZMIZ2) involved in Wnt1/ β -catenin signaling in colorectal cancer and papillary thyroid cancer^{62,63}; Netrin B receptor (UNC5B), which is involved on Netrin-1 signaling in gastric cancer cell proliferation via PI3K/AKT pathway⁶⁴; Disk, Large homolog-associated protein 2 (DLGAP2) is involved in pancreatic cancer through AMPK pathway⁶⁵; G protein-couple receptor (GPR176) is implicated in breast cancer transcriptional response,⁶⁶ which is an immune therapy target for ovarian cancer^{67,68}; and Lysine-specific histone demethylase 5C (KDM5C) gene that promotes prostate cancer by repressing PTEN expression.⁶⁹

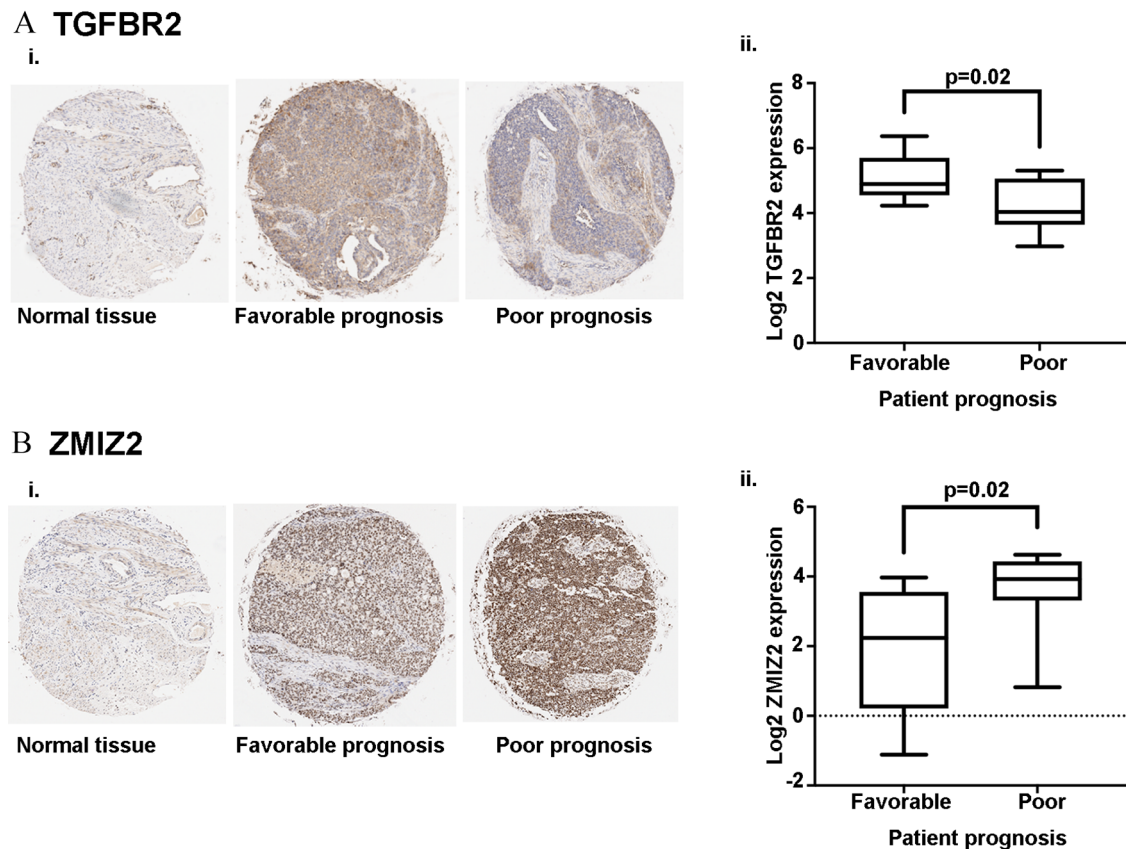


Figure 5. Expression of higher dosage genes in poor prognosis patients compared to favorable prognosis patient tumors. (A) TGFBR2 overexpression in patients, (i) Representative IHC staining of TGFBR2 in the FFPE tissue sections at 4× magnification, (ii) Expression levels of TGFBR2 on the cell membranes of the cells in tumor tissues of the ovarian cancer patients. (B) ZMIZ2 overexpression in patients, (i) Representative IHC staining of ZMIZ2 in the FFPE tissue sections at 4× magnification, (ii) Expression levels of ZMIZ2 in the nucleus of the cells in tumor tissues of the ovarian cancer patients.

Analysis of these 15 genes in TCGA database oncoprint (National Cancer Institute, The Cancer Genome Atlas) using cBioportal,^{70,71} further confirmed that these genes were predominantly amplified, or deleted but not mutated (Supplemental figure 2). These gene amplification/deletions variation among patients suggested that their functional protein expression may vary in the patient tumor samples.⁷² Hence, the top 2 proteins identified by Reactome functional database analysis that is, TGFBR2 and ZMIZ2 were tested for their protein expression in the patient tumor samples. About 3% of patients reported in TCGA data show amplification and our sequencing results show higher dosage of TGFBR2 in poor prognosis patients. In contrast, the protein expression, as monitored by TMA, was downregulated in poor prognosis patient tumor samples, thus presenting an apparent inconsistency between the data from the cfDNA analysis and TMA analysis. It should be noted here that TGFBR2 shows increased expression in omental metastases and invasive front of ovarian cancer.⁵¹ Since our TMA does not represent any samples from omental metastases or invasive front of ovarian cancer tissue, it is more likely that our TMA analysis missed the expression profile of TGFBR2. Previous findings that the expression of TGFBR2 is closely associated with ovarian cancer progression also point to a prognostic role for TGFBR2. Further IHC analysis of TMA representing

different locales of ovarian cancer tissues should establish the unequivocal role of TGFBR2 as a prognostic biomarker in ovarian cancer liquid biopsy. It should also be noted here that the age difference between the poor-prognosis and favorable-prognosis is significantly different (Table 1). Considering the previous findings that omental metastases show increased expression of TGFBR2,⁵¹ it is possible that the age difference and possibly the associated metastatic stage of the tumor plays a role in presenting TGFBR2 as a cfDNA-based prognostic biomarker in ovarian cancer. Thus, it is possible that age could be a parameter in deciding which biomarker can be used for prognosis. Further analysis with distinct age group, histological sub-types, stage/grade, and spatial localization of the expressed markers is anticipated to consolidate the prognostic biomarker candidacy of cfDNA-gene signatures such as that of TGFBR2.

Interestingly, gene signature for ZMIZ2 shows higher dosage as well as protein upregulation in poor prognosis patient tumor samples. This corroborates well with the previous findings that indicated a pro-tumorigenic role for ZMIZ2.^{73,74} Staining of ZMIZ2 on a second cohort of ovarian cancer patients, reiterated the upregulation of ZMIZ2 in poor prognosis patients. Functionally, this is validated by the findings that the expression of ZMIZ2 is correlated with the Wnt/beta-catenin signaling⁷⁴ and Wnt-signaling is often dysregulated in

ovarian cancer.⁷⁵ In light of its tumor promoting role in other cancers,^{62,76,77} ZMIZ2 could prove to be a valid prognostic biomarker for ovarian cancer poor prognosis/therapy-resistance.

Various oncogenes and tumor suppressors have been identified as prognostic and diagnostic biomarkers for ovarian cancer progression.⁷⁸ cfDNA based biomarkers are investigated widely for methylation patterns and DNA copy number variations in ovarian cancer patients.^{79,80} Advances in next generation sequencing allowed our approach to sequence the minimal quantity of cfDNA for gene signature panels.⁸¹ Although, the level of amplification of the considered genes may be low, this pilot project indicates presence of specific genes as cfDNA fragments in serum and respective protein expression levels in tumor tissue, which of those can be exploited further as multifactorial definition for prognosis determination in ovarian cancer patients. However, we acknowledge that larger cohort of patients, inclusive of various ethnic background, need to be tested and an unequivocal panel of gene signatures developed to serve as a novel tool for stratification of prognosis among ovarian cancer patients.

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Data availability

All the data generated for this publication are included in the paper. The sequencing data generated during the current study are available in the NCBI SRA repository, <https://www.ncbi.nlm.nih.gov/sra/PRJNA517159>.

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Supplemental material

Supplemental material for this article is available online.

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