Prostaglandin D2 expression is prognostic in high-grade serous ovarian cancer

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Received March 12, 2018; Accepted August 2, 2018

DOI: 10.3892/or.2019.6984

Abstract. To identify biomarkers that could predict response or lack of response to conventional chemotherapy at the time of diagnosis of high-grade serous ovarian carcinoma (HGSOC), the present study compared large-scale gene expression from patients with short or long disease-free survival times, according to the last cycle of chemotherapy, and validated these findings using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and conventional immunohistochemical (IHC) analysis. Samples were selected for microarray evaluation, at the time of diagnosis, using the following criteria: Identical debulking primary surgery, International Federation of Gynaecology and Obstetrics staging, histological subtype and grade. These were divided into 2 groups, regarding the outcome after 2 years of follow-up. Prostaglandin D2 synthase 21 kDa (brain) (*PTGDS*) was found to be expressed at a significantly higher level in the tumours of patients with a short disease-free survival time, and this was validated by RT-qPCR in all samples. Furthermore, the study evaluated PGD2, the protein product of the *PTGDS* gene, in a large cohort of 114 HGSOC patients using the Ventana Benchmark automated platform, and IHC positivity was correlated with clinicopathological data and outcome. The global gene expression analysis identified 1,149 genes that were differentially expressed in microarray data, according to the patient outcome. Further analysis RT-qPCR validated *PTGDS* gene expression in the same samples (r=0.945; P<0.001). IHC analysis showed an inverse profile, with positivity for PGD2 strongly associated with an increase in disease-free survival (P=0.009), the absence of relapse (P=0.039) and sensitivity to platinum-based therapy (P=0.016). Multiple Cox regression showed that IHC evaluation of PGD2 was also a prognostic marker associated with relapse (hazard ratio, 0.37; P=0.002). Overall, the results showed that IHC evaluation of PGD2 is an independent marker of good prognosis in HGSOC. This finding contributes to our understanding of the mechanism of tumour regulation and to investigations into biomarkers that predict response to chemotherapy.

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Key words: high-grade serous ovarian carcinoma, microarray, gene expression, reverse transcription-quantitative polymerase chain reaction, immunohistochemistry, prognosis

Introduction

Ovarian cancer, which ranks fifth in terms of cancer-associated mortalities among women, is the most lethal gynaecological malignancy (1). Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer and comprises a heterogeneous group of diseases. High-grade serous ovarian carcinoma (HGSOC), which is the most common EOC subtype, has several unique clinical features and high genomic instability (2). Irrespective of the histological type, the standard first-line chemotherapy for EOC is platinum-based either as a single agent or in combination with paclitaxel (3). However, there is evidence that response to this therapy varies in different histological subtypes (4).

Historically, the estimation of prognosis for a number of cancer sites relied heavily on conventional clinicopathological parameters. More recently, molecular profiling has provided an additional dimension to improve prognostic accuracy and has assisted in identifying subclasses of tumours more closely linked to prognosis. Potentially, such an approach can also reveal novel targets for treatment (5).

Prostaglandins (PGs) are a family of biologically active endogenous metabolites of arachidonic acid. PGs control a large range of physiological functions, including the regulation of smooth muscle tone, inflammation, cellular growth and differentiation (6). In the normal ovary, PGD2 signalling interferes with the action of follicle-stimulating hormone within granulosa cells, thereby indicating an important role for PGD2 signalling in the modulation of the balance of the proliferation, differentiation and steroidogenic activity of these cells (7). The protein product of prostaglandin D2 synthase 21 kDa (brain) (*PTGDS)*, PGD2 protein, was initially identified as the main prostaglandin in the brain (8) and within brain tumours (9). PGD2 has previously been shown to inhibit cell migration and invasion (10-14). Reduction in the protein expression of PGD2 was shown to be a significant biological event that is involved in the malignant progression of astrocytomas and predicts poor patient survival (12). In addition, PGD2 levels in serous cystadenocarcinoma of the ovary were shown to inhibit *in vitro* and *in vivo* ovarian cancer cell growth in a dose-dependent manner, subsequently extending the survival time of nude mice with these tumours (10).

In order to identify markers that could predict response to conventional therapy at the time of first surgery, an exploratory large-scale gene expression array was conducted in a cohort of 11 patients with HGSOC in the present study, and whether the expression of these genes could be used to predict response in a larger cohort of 114 HGSOC FFPE samples with a known outcome was assessed. In the cohort of 114 HGSOC patients, PGD2 evaluation by immunohistochemistry (IHC) was found to be a marker of a good prognosis.

Patients and methods

Tissue sample collection for microarray analysis. The study design was approved by the Medical Ethical Committee of the A.C. Camargo Cancer Center (São Paulo, Brazil; 1863/14). For this first approach, patients were selected who were diagnosed with an advanced stage of HGSOC between January 2000 and August 2013, and whose specimens were obtained from optimal debulking surgery (<1 cm of gross residual disease) (15) prior to chemotherapy. All patients provided written informed consent for the collection of samples and subsequent analysis, and this study was approved by the Ethics Committee of the A.C. Camargo Cancer Center (1863/14). All patients with advanced-stage HGSOC were treated with platinum-taxane standard chemotherapy following surgery (6 cycles of carboplatin with area under the curve of 5 or 6, and 175 mg/m^2 paclitaxel on day 1 every 21 days). Cancer antigen 125 (CA125) serum level and image analyses were routinely performed at follow-up visits. Patients with unavailable data regarding primary surgery and chemotherapy, and patients with a follow-up time of <24 months were excluded for the analysis of platinum response. A total of 11 frozen primary HGSOC tissue specimens were selected from the available samples in the institution biobank following these criteria.

Clinical endpoints. For the survival signature, disease-free survival was calculated as the interval from primary surgery to disease progression or recurrence. The platinum-free interval (PFI) was considered the interval between the date of the last platinum compound infusion and the date of disease progression. In a cohort of 114 HGSOC patients, the protein expression was evaluated along with anatomopathological data and chemotherapy response. Cases in which patients relapsed within 6-12 months were defined as platinum partially sensitive. Patients who relapsed >12 months later were termed platinum-sensitive and those who relapse within 6 months of completing initial treatment was classified as being platinum-resistant (16,17). Progression was defined per the Gynaecological Cancer InterGroup (GCIG) criteria (18) following the evaluation of Response Evaluation Criteria In Solid Tumors and CA125 progression in the medical charts, and the date of the earlier event was considered for progression. GCIG considers CA125 progression to have occurred if there is a doubling in CA125 from the upper limit of the normal range (normal range, 0.6-35.0 U/ml) (19). For those patients whose CA125 level does not decrease to within the normal range, a doubling from the nadir is considered progression (20,21). Overall survival was determined by the time interval between the date of diagnosis and the date of mortality due to ovarian cancer.

Sample processing and gene expression profiling. Upon histological evaluation, frozen tissues containing tumour cells were used for RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocols. The RNA quality was assessed using the RNA 6000 Nano kit on the Agilent 2100 Bioanalyzer Platform (Agilent Technologies, Inc., Santa Clara, CA, USA). Only samples with an RNA integrity number >7 were considered suitable for the reaction. Total RNA (100 ng) was converted into labelled cRNA. Universal Human Reference RNA was labelled with Cy3 and used to control for variability in array hybridization, establishing the same denominator of data analysis in the reaction. Tumour cRNA was labelled with Cy5 and hybridized for 17 h at 65˚C to an Agilent Whole Human Genome Oligo Microarray 4x44K (G4112F; Agilent Technologies, Inc.). The hybridized microarray was washed and then scanned in a microarray scanner with Microarray Scanner System (Agilent Technologies, Inc.), with Scan Control Software 8.1 (Agilent Technologies, Inc.). Signal intensity per spot was generated from the scanned image with Feature Extraction Software with default settings. The microarray data were normalised by intensity-dependent global normalisation (locally weighted scatterplot smoothing) using the Agilent Feature Extraction Software (v.10.1.1.1; Agilent Technologies, Inc.). The raw data were normalised by median-centring the genes for each array and then log2 transformation. The Limma package (22) was used to obtain differentially expressed genes and the statistical comparison between samples with different clinical responses was performed using the R package, considering $P<0.05$ as indicating a statistically significant difference.

In order to identify upregulated or downregulated genes in a comparison of two different RNA populations, a threshold value defined in relation to fold-change was used, where ≥4.0 and ≤-4.0 was used to classify the most and least regulated genes, respectively. To compare the microarray datasets measured in this group of tumour samples, patterns of gene expression were compared between patients without disease

recurrence after 2 years from their last platinum treatment, considered as 'long-term disease-free patients' and patients with recurrence within 2 years from the last platinum treatment following initial surgery and first-line treatment, considered as 'short-term disease-free patients'. This cut-off was established according to previous studies showing that the average patient experienced clearly defined progression within 18-24 months of cisplatin/paclitaxel therapy (23,24).

The differentially expressed genes were submitted to *in silico* analysis of biological function, canonical pathway and upstream regulator analysis interaction networks using the Ingenuity Pathway Analysis software (IPA; v8.0; Ingenuity® Systems, Redwood City, CA, USA; http://www.ingenuity.com), and for graphical representation, the TMeV v4.8 program (www.tm4.org) was used to construct the heatmap to obtain a gene expression profile. Hierarchical clustering was performed using Euclidean distance. In parallel, the differentially expressed genes were searched for significantly enriched pathways with the software KOBAS 2.0 (25). Only pathways identified simultaneously by IPA and KOBAS with $P \leq 0.05$ were considered for further interpretation.

Technical validation using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to evaluate the reproducibility of microarray data through transcript quantification, RT-qPCR technology was used as a support technique to validate and quantify the drivers. Phospholipase C β2 (*PLCB2)* and *PTGDS* were selected for validation by RT-qPCR, taking into account *in silico* analyses by IPA software together with an accurate search in the literature and the biological role of these genes in the context of HGSOC. The RNA samples used in the microarray analysis were converted into cDNA from 2 μg total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), following the manufacturer's protocols. RT-qPCR was performed in triplicate on Applied Biosystems 7500HT Fast Real-Time PCR system equipment (Applied Biosystems; Thermo Fisher Scientific, Inc.) with initial denaturation at 95˚C for 3 min, followed by a second denaturation at 95˚C for 30 sec and annealing at 60˚C for 30 sec for 40 cycles. The assays were performed using SYBR®-Green Master Mix (Thermo Fisher Scientific, Inc.) following the manufacturer's protocols and the quantitation cycle (cq) values were submitted to a $2^{-\Delta\Delta Cq}$ analysis (26). The Universal Human Reference RNA (cat. no. 740000; Agilent Technologies, Inc.) was used as reference sample, and as endogenous controls, glyceraldehyde phosphate dehydrogenase, β-actin (*ACTB*) and *18S rRNA* were tested, and thereafter, *ACTB* was selected as the endogenous control due to its lower threshold cycle variation among the samples. All the primer sequences are summarized in Table I.

Protein expression by IHC using tissue microarrays (TMA). Histological slides were reviewed by an experienced gynaecological pathologist in order to select the most representative paraffin block from each tumour. Histological subtype was revised based on World Health Organisation classification of ovarian tumours (27). The degree of histological differentiation was determined according to Malpica classification (28). For TMA construction, two representatives of 0.6-mm tumour

Table I. Oligonucleotides sequence and reverse transcription-quantitative polymerase chain reaction conditions.

Gene	Forward	Reverse	Amplicon	Slope	Efficiency	Primer concentration, nM
PLCβ2	CAGACAAGATGGCCCAGGAG	CTCCCGTATCTGTTCCAGGC	155	-3.19	1.05	400
PTDGS	CTTCCTGCCCCAAACCGATA	GCAGAGACATCCAGAGCGTG	106	-3.451	0.95	200
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	110	-3.529	0.92	25
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA	107	-3.448	0.94	50
ACTB	TCCCTGGAGAAGAGCTACGA	AGCACTGTGTTGGCGTACAG	90	-3.309	1.00	100

PLCβ2, phospholipase C β2; *PTDGS*, prostaglandin D2 synthase; *GAPDH*, glyceraldehyde phosphate dehydrogenase; *ACTB*, β-actin.

Table II. Samples selected for large-scale gene expression analysis.

Sample no.	Age at diagnosis, years	RIN	Stage	Disease-free survival, months	Microarray analysis
1	55	9.1	IIIC	23.9	Long-term disease-free patients
2	45	8.8	IIIC	13.6	Short-term disease-free patients
3	43	9.1	IIIC	8.9	Short-term disease-free patients
4	43	5.1	IIIC	8.3	Short-term disease-free patients
5	52	9.2	IIIC	2.0	Short-term disease-free patients
6	58	8.6	IIIC	22.5	Long-term disease-free patients
7	69	6.4	IIIC	6.3	Short-term disease-free patients
8	71	5.1	IIIC	20.9	Short-term disease-free patients
9	58	5.4	IIIB	21.2	Short-term disease-free patients
10	68	6.5	IIIC	34.6	Long-term disease-free patients
11	45	8.9	IIIC	14.0	Short-term disease-free patients

RIN, RNA integrity number; short-term disease-free patients, patients with disease recurrence within 2 years; long-term disease-free patients, patients with a platinum-free interval of >2 years.

cores were embedded on a recipient paraffin block. Once the whole recipient block was finished, it was baked at 42˚C for 40 min and mapped in a spread table for subsequent evaluation.

Immunohistochemical analysis was performed on 5-mm thick TMA sections using a polyclonal antibody against PGD2 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-201221A) at 1:100 dilution followed by Ventana detection system in a Discovery XT automated instrument (Ventana Medical Systems, Inc., Tucson, AZ, USA). The antibodies were incubated at 37˚C for 32 min and the complex was then visualized with hydrogen peroxide substrate and 3,3'-diaminobenzidine tetrahydrochloride chromogen and counterstained with hematoxylin. All batches included cerebral cortex, provided from the samples archives of the AC Camargo Cancer Center, as positive controls, and omission of the primary antibody was used as a negative control.

Slide digitization and IHC analysis. Digital images of IHC-stained TMA slides were obtained at x20 magnification using a Panoramic 250 High Throughput Scanner (3DHistech, Budapest, Hungary). Images were loaded and visualized using a specialized server (Panoramic Viewer). PGD2 expression was noted as sharp, well-localized cytoplasmic staining and the expression pattern was evaluated in a quantitative manner, whereby the levels of expression were represented by the intensity of staining and was scored on a scale of 1+ to 3+. All sections were analysed in a blinded manner. For the descriptive statistics, association tests and survival analysis, samples were classified into 2 groups, namely those that exhibited weak expression of PGD2 and those that more strongly exhibited PGD2 (2+ and 3+) (29).

Statistical analysis. Statistical analysis was performed using SPSS version 22.0 (IBM, Corp., Armonk, NY, USA) for Windows. The association between categorical variables was analysed by χ^2 or Fisher's exact tests. Correlation among numerical variables was determined by Spearman's correlation test for non-normally distributed data. In a cohort of 114 patients used to evaluate PGD2 expression, survival curves were constructed by Kaplan-Meier analysis, and the comparison of survival curves for each variable category was performed using a log-rank test. Univariate and multivariate analyses were performed using Cox regression. For multivariate analysis, all variables with $P<0.20$ in the univariate analysis were entered into the test (30). For all tests, $P\leq0.05$ was considered to indicate a statistically significant difference.

Results

cDNA microarray analysis. Whole tumour gene expression profiling was conducted on 11 patients with HGSOC, ranging in age from 42 to 68 years (median, 52 years), as shown in Table II. The median follow-up time was 59 months, ranging from 21.40 to 98.08 months. A previous study showed that among patients who present with advanced disease, 10-15% achieve long-term remission; however, the remainder tends to undergo a progression of treatments (31). The number of selected patients for the initial approach in the present study were within this range and showed a PFI of 21 months, with 8 patients (73%) who underwent a relapse within 2 years (group 1: Short-term disease-free patients) and 3 patients (27%) who were characterized as long-term disease-free patients (group 2). Using these 2 groups, the global gene expression was obtained, and observations from the heat map (Fig. 1) showed a molecular similarity between samples according to clinical response. The colour distribution on the heat map assisted in aggregating the data visually, aiding the detection of distribution patterns.

Applying a threshold value, defined in relation to fold-change |4.0|, 150 differently expressed genes, 97 of which were downregulated and 53 of which were upregulated, were acquired (data not shown). Enrichment analysis using two methods (IPA and KOBAS) identified pathways that were significantly over-represented ($P<0.05$) among the 150 differentially expressed genes (Table III). The enrichment analysis

Table III. Gene enrichment analyses using Ingenuity Pathway Analysis and KOBAS 2.0 tools pathways of the 150 selected genes.

Molecular mechanisms of cancer	Pathway	Database	ID	P-value
Cell division and survival	Cell cycle	Reactome	REACT_111214	0.040
	Regulation of extrinsic apoptotic signalling pathway via death domain receptors	Gene Ontology	GO:1902041	0.027
	Regulation of execution phase of apoptosis	Gene Ontology	GO:1900117	0.032
	p53 signalling pathway	KEGG PATHWAY	hsa04115	0.040
Invasion	TGF-β receptor signalling in EMT	Reactome	REACT_120726	0.019
	Regulation of cell migration	Gene Ontology	GO:0030334	0.006
	Cell-matrix adhesion	Gene Ontology	GO:0007160	0.014
	Regulation of cellular component movement	Gene Ontology	GO:0051270	0.024
	Cell junction organization	Gene Ontology	GO:0034330	0.042
	Focal adhesion assembly	Gene Ontology	GO:0048041	0.047
Regulatory mechanisms	mRNA transcription from RNA polymerase II promoter	Gene Ontology	GO:0042789	0.017
	Drug binding	Gene Ontology	GO:0008144	0.041
	Positive regulation of NF-κB import into nucleus	Gene Ontology	GO:0042346	0.044
	ER-nucleus signalling pathway	Gene Ontology	GO:0006984	0.049
Control of cell growth	Regulation of SMAD protein import into nucleus	Gene Ontology	GO:0060390	0.019
	Insulin receptor binding	Gene Ontology	GO:0005158	0.011
	Cellular response to gonadotropin stimulus	Gene Ontology	GO:0071371	0.029
Angiogenesis	Angiogenesis involved in wound healing	Gene Ontology	GO:0060055	0.019
	Positive regulation vascular endothelial growth factor production	Gene Ontology	GO:0010575	0.044
Metabolism	Arachidonic acid metabolic process	KEGG PATHWAY	hsa00590	0.007
	Membrane lipid metabolic process	Gene Ontology	GO:0006643	0.011
Interaction with immune system	Granulocyte migration	Gene Ontology	GO:0097530	0.027

GO, Gene Ontology; KEGG, Kyoto Encyclopaedia of Genes and Genomes.

performed using the IPA software paired with the enrichment analysis of the KOBAS software generated 22 annotations of pathways associated with the molecular mechanisms of cancer, some of which were associated with mechanisms of 'cell division and survival', 'invasion', 'regulatory mechanism', 'growth', 'angiogenesis', 'metabolism' and 'interaction with the immune system' (Table III). The 'p53 signalling pathway', a tumour suppressor protein pathway that is the most commonly mutated in human cancer, along with a high frequency of alteration in high-grade serous adenocarcinoma (32), appeared to negatively regulated in the present data analysis (IPA, P<0.001; KOBAS, P=0,04) within lipid metabolism. This illustrates the key role played by lipids in maintaining homeostasis, which was also altered for molecular function in IPA software (P≤0.001) and in the KOBAS database (characterized as the regulation of 'membrane lipid metabolic process', Gene Ontology: 0019216, P=0.03, and 'arachidonic acid metabolic process', Kyoto Encylopaedia of Genes and Genomes pathway, P=0.0072).

Validation of the selected transcripts by RT-qPCR. RT-qPCR analysis was used to confirm transcriptome alterations detected by microarray in all 11 selected samples. Constitutive expression of *ACTB* was used to correct variations and difference between RNA quantification (Table I). The average fold-change value was extracted from each sample of the microarray assay and the RT-qPCR in order to evaluate the correlation between the values obtained by the two techniques. *PLCB2* and *PTGDS* belonging to the metabolism of lipids pathway were submitted to technique validation and *PTGDS* showed a significant correlation between the results of microarray and RT-qPCR (Table IV).

IHC analysis. Based on validation by RT-qPCR, the protein product of *PTGDS*, PGD2, was chosen for further studies with IHC. A larger set of HGSOC samples was available as formalin-fixed paraffin-embedded sections for this analysis, providing 114 specimens for protein evaluation and correlation with clinicopathological data and survival rates. The

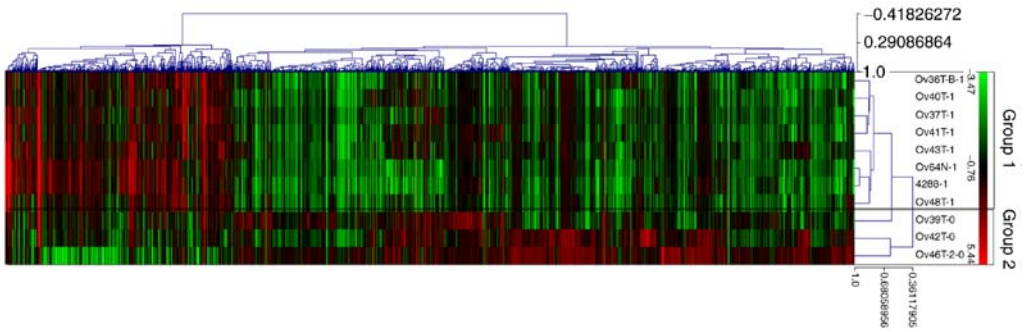

Figure 1. A representation of unsupervised cluster analysis based on the global expression genes list of microarray analysis (n=1,149). Samples consist of patients with disease recurrence within 2 years (group 1) and samples of patients with a platinum-free interval of 2 years (group 2), showing a cluster tendency together with a high number of hyper-regulated genes. Green colouration corresponds to downregulated genes and red colouration corresponds to upregulated genes.

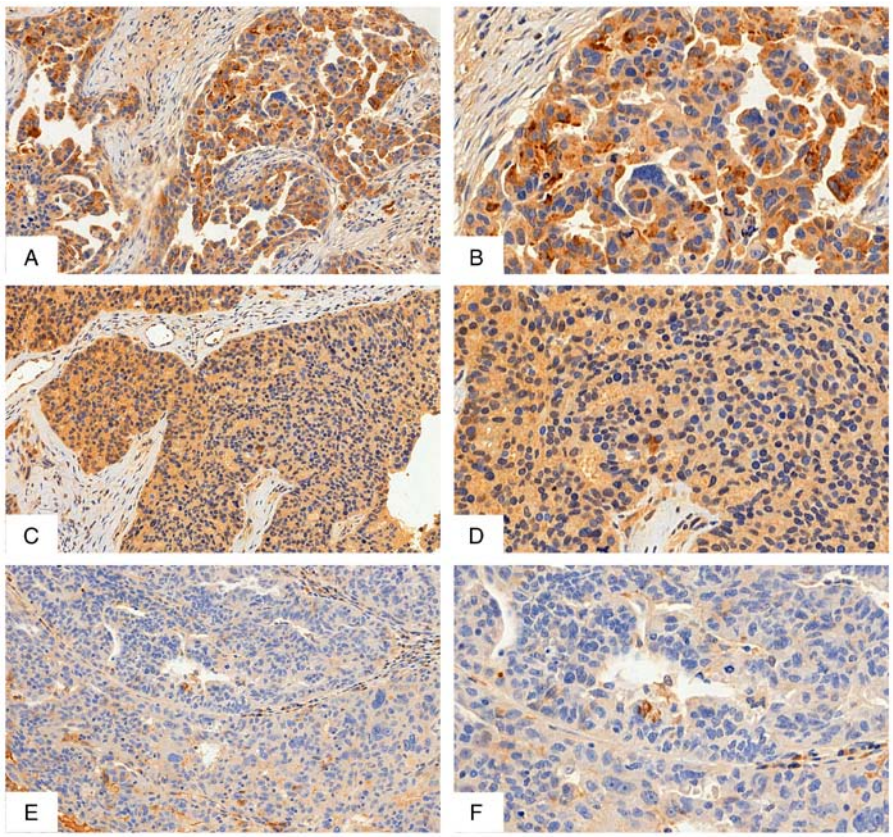

Figure 2. Marker expression by immunohistochemistry of PGD2 (n=114) according to patient survival time. (A and B) Representative images from patients with no relapse at 5 years of follow-up, showing strong expression of PGD2 (3+ intensity). (C and D) Representative images from patients who relapsed within 18 months of follow-up (belonging to the 'long-term disease-free patients' group) (2+ of intensity). (E and F) Weak expression of protein on high-grade serous ovarian carcinoma (1+ of intensity) (belonging to the 'short-term disease-free patients' group). (A, C and E) x20 and (B, D and F) x40 magnification. PGD2, prostaglandin D2 protein.

Table IV. Fold-change of genes drivers obtained by microarray and RT-qPCR and its correlation.

	Fold-change						
	Microarray		RT-qPCR		Technique validation		
Gene	Group 1	Group 2	Group 1	Group 2	95% CI	Correlation	P-value
PLCB2	3.08	-0.22	2.71	-0.41	-0.4404-0.7418	0.236	0.484
PTGDS	4.30	1.63	6.15	1.60	0.7908-0.9866	0.945	<0.001[a]

[a]Statistically significant. Correlation among numerical variables determined by Spearman's correlation test. CI, confidence interval; group 1, patients with disease recurrence within 2 years; group 2, patients with a platinum-free interval of 2 years; *PLCB2*, phospholipase C β2; *PTGDS*, prostaglandin D2 synthase 21 kDa (brain); RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

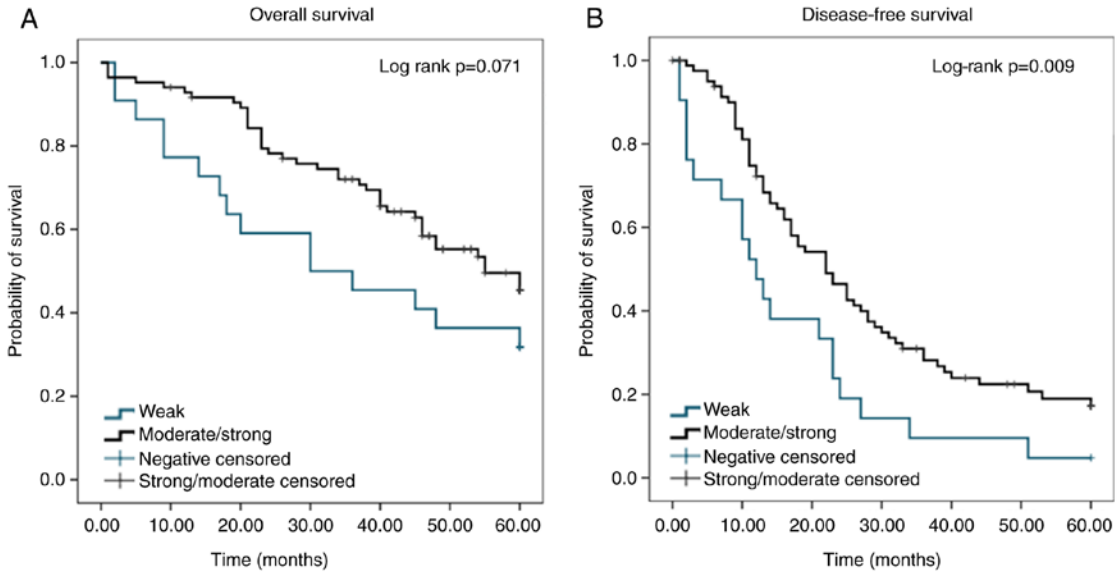

Figure 3. Kaplan-Meier curve for (A) overall and (B) disease-free survival with regard to prostaglandin D2 protein evaluated in 114 patients, where weak represents 1+ intensity on immunohistochemical evaluation and moderate/strong represents 2+ and 3+ (categorized together for this analysis). All P-values were calculated by the log-rank test.

median age of this cohort was 59 years, ranging from 33 to 82 years. A total of 97 (88.2%) patients out of 110 women who had this information in the medical charts were diagnosed in advanced-stages (FIGO staging III-IV), 91 out of 113 (80.5%) relapsed, 50 out of 114 (43.9%) were alive at the time of the study and 30 out of 107 patients (28.0%) were platinum-mjresistant. Clinicopathological features of all 114 patients are described in Table V. PGD2, a prostaglandin associated with suppression of inflammation is visualised by IHC as well-defined cytoplasmic staining (Fig. 2). According to χ^2 and Fischer's exact tests, the lower intensity of PGD2 was associated with mortality (P=0.05), relapse (P=0.039) and resistance to platinum chemotherapy (P=0.016) (Table VI).

Kaplan-Meier survival analysis and multivariate Cox regression analysis of patient outcomes. In a cohort of 114 patients used to evaluate PGD2 expression, survival curves (Fig. 3) were created to show the association between PGD2 expression and outcome. The median disease-free survival for patients whose tumours exhibited moderate to strong expression of PGD2 was 22 months, which was significantly higher than the median survival of patients whose tumours exhibited weak PGD2 expression (12 months) (P=0.009). A Cox proportional hazards regression model showed that a score of 1+ for PGD2 protein expression in the tumour was a strong predictor of poor disease-free survival [hazard ratio (HR), 0.52; 95% confidence interval, 0.31-0.86; P=0.01]. The prediction of mortality was not statistically significant (HR, 0.58; 95% confidence interval, 0.32-1.06; P=0.08).

Putative prognostic variables with P<0.20 in the univariate analysis (data not shown) were entered into a multivariate analysis to identify independent prognostic factors. Multivariate analysis showed a significant association between a lower level of PGD2 expression and a high risk of progression following chemotherapy (HR, 0.37; 95% CI, 0.20-0.69; P=0.002) (Table VII).

Table V. Clinicopathological features of 114 patients with high-grade serous ovarian carcinoma analysed in formalin-fixed, paraffin-embedded samples.

Variables	Category	n/total n (%)
Staging	I/II	13/110 (11.8)
	III/IV	97/110 (88.2)
CA125 (median/range)	<780	46/91 (50.5)
	>780	45/91 (49.5)
ECOG PS	0	40/89 (44.9)
	1, 2, 3	49/89 (55.1)
Age, years	<59	55/114 (48.2)
	>59	59/114 (51.8)
Mortality	No	50/114 (43.9)
	Yes	64/114 (56.1)
Relapse	No	22/113 (19.5)
	Yes	91/113 (80.5)
Platinum response	Platinum-resistant	30/107 (28)
	Partially platinum-sensitive	20/107 (18.7)
	Platinum-sensitive	20/107(18.7)
	Responder	37/107 (34.6)

ECOG PS, Eastern Cooperative Oncology Group performance status; CA125, cancer antigen 125.

Discussion

Disease heterogeneity is an under-appreciated challenge in pre-diagnostic biomarker identification. Molecular analysis is increasingly revealing that diseases that were once considered monotypic are actually multiple molecular diseases sharing a

Table VI. Distribution of high-grade serous ovarian carcinoma samples according to intensity of prostaglandin D2 protein and standard prognostic factors.

Variables	Category	+1, n (%)	2+/3+, n (%)	P-value
Staging	I/II	1 (4.8)	12 (14.6)	0.296[a]
	III/IV	20 (95.2)	70 (85.40	
Pre-treatment CA125 (median/range)	>780	5 (33.3)	39 (55.7)	0.197[b]
	<780	10 (66.7)	31 (44.3)	
Age, years	<59	12 (54.5)	42 (50)	0.889[b]
	>59	10 (45.5)	42 (50)	
ECOG PS	0	5 (27.8)	35 (53)	0.102[b]
	1, 2, 3	13 (72.2)	31 (47)	
Mortality	No	5 (22.7)	41 (48.8)	0.05[b,c]
	Yes	17 (77.3)	43 (51.2)	
Relapse	No	1 (4.5)	21 (25)	0.039[a,c]
	Yes	21 (95.5)	64 (75)	
Platinum response	Platinum-resistant	11 (55)	16 (20)	0.016[a,c]
	Partially platinum-sensitive	2 (10)	17 (21.3)	
	Platinum-sensitive	4 (20)	16 (20)	
	Responder	3 (15)	31 (38.8)	

[a]Fisher's exact test; [b]χ^2; [c]P≤0.05. ECOG PS, Eastern Cooperative Oncology Group performance status; CA125, cancer antigen 125.

Table VII. Estimation of parameters of multiple Cox regression model for mortality and relapse in the tissue microarray cohort.

	Mortality		Relapse	
Feature	P-value	HR (95% CI)	P-value	HR (95% CI)
Surgery	0.003[a]	3.61 (1.53-8.48)	0.002[a]	2.50 (1.39-4.48)
Residual disease	0.004[a]	3.12 (1.63-5.99)	<0.001[a]	2.55 (1.5-4.34)
ECOG PS	0.003[a]	3.01 (0.17-1.15)	-	-
PGD2	-	-	0.002[a]	0.37 (0.20-0.69)

[a]P≤0.05. Independent variables used on this analysis: Age, surgery, residual disease, staging and ECOG PS. HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; PGD2, prostaglandin D2 protein.

common clinical presentation (33). Ovarian cancer is no exception and shows a huge genomic complexity among its different subtypes. A molecular approach to ovarian cancer allows us to investigate biological differences that are not otherwise visible through morphology, and provides an indication of the potential impact of genomics on cancer research and care. In the present study of ovarian cancer, a set of 11 samples were carefully selected according to well-established prognostic factors in order to understand how samples with the same morphological features could have such different clinical outcomes from the same treatment.

An *in-silico* analysis using IPA software combined with the KOBAS database showed that the drivers significantly expressed in the microarray platform were strongly involved in molecular mechanisms of cancer. The enrichment analysis that was undertaken for the gene list demonstrated their participation in main networks that regulate several functions of cancer development and progression, thus corroborating the selection of potential drivers. Metabolic reprogramming is now established as a hallmark of cancer (34) and metabolites that change significantly provide insights into the biochemical consequences of transformation and could be considered candidate biomarkers of ovarian carcinogenesis (35). In order to confirm our large-scale gene expression results, *PTGDS* was selected and validated based on the metabolism of lipids pathway.

PGs are a family of arachidonic acid and metabolites, with specific roles for different proteins. Certain PGs exhibit pro-carcinogenic effects, for example, PGE2, which is associated with the proliferation of various types of cancer (36-38), whilst others, such as PGD2, can exhibit cancer-protective

characteristics (12,39). This enzyme-derived metabolite is known to act as a negative feedback regulator of cyclo-oxygenase-2 (COX-2), and has been reported to act as a 'landscaping tumour promoter' (40). In a previous study, the PGD2 metabolite 15d-PGJ2 suppressed the cytokine-induced expression of COX-2 by peroxisome proliferator-activated receptor γ (PPARγ) in a PPARγ-dependent and -independent manner. PPARγ is a member of the nuclear hormone receptor superfamily and functions as a ligand-dependent transcription factor (41). PPARγ/RXR heterodimer activated by 15d-PGJ2 upregulates transcription of IκB, which stops nuclear factor-κB (NF-κB) from activating COX-2 transcription. Additionally, PPARγ agonists can inhibit IκB kinase (IKK) in the cytosol, thus preventing IκB phosphorylation, leading to the nuclear translocation and activation of NF-kB, and thereby the suppression of COX-2 gene transcription (42,43).

Certain studies previously evaluated mRNA in EOC samples and reported that *PTGDS* was involved in disease progression (44,45). Kaplan-Meier Plotter, a web application for assessing the effect of gene expression and survival rates using cancer samples (http://kmplot.com/analysis/index.php?p=service), including HGSOC mRNA samples, showed that *PTGDS* mRNA expression was associated with a worse prognosis in terms of disease-free survival (HR, 1.5; P=0.0146). However, it is unwise to focus on isolated information based on gene expression only.

It has been common practice to use mRNA concentrations to deduce concentrations and activities of the corresponding proteins, but this assumes that transcript abundance is the main determinant of protein abundance. However, transcriptional and post-transcriptional regulation, for example by microRNA (miRNA/miR), cannot be ignored (46). During an investigation on miRNA roles in tongue squamous cell carcinoma cell lines and a cisplatin-resistant cell line, Yu *et al* (47) reported that miR-518c, and miRNA that has *PTGDS* as a putative target, was differentially expressed, with a high level of expression in the cisplatin-resistant cell line. This suggested that *PTGDS* transcripts may be regulated by miRNAs, and may explain the discrepancy observed in the present results, in which mRNA levels do not follow the same protein level pattern.

In the present study, the expression of PGD2 evaluated by IHC was associated with an improved prognosis, and thus represented a potential molecular target involved in determining the clinical outcome in patients with HGSOC. A study by de Jong *et al* (47) in an ovarian cancer cell line demonstrated the effects of PGD2 metabolite 15d-PGJ2 on apoptosis, cell migration, transformation and drug resistance, indicating that 15d-PGJ2 may reduce drug resistance and inhibit tumour metastasis by inhibiting NF-κB. These results aid in improving our understanding of the complex actions of the endogenous metabolite PGD2, suggesting its potential therapeutic use as an anticancer agent (48).

Ovarian cancer is the most common cause of gynaecological cancer-associated mortality among women in developed countries. However, certain subgroups of patients experience comparatively longer survival times. Research is currently being performed for the identification of prognostic factors that characterize such patients, and areas of investigation include biomarker studies. Key aspects of biomarker development include careful study design and sample selection to avoid bias, comprehensive testing, validation and accurate reporting of the results (49). The present study provides evidence that IHC evaluation of PGD2 protein in HGSOC surgical samples generates prognostic information in patients treated with a standard approach, such as cisplatin or carboplatin, and a taxane, such as paclitaxel. These findings can be used for patient assessment in multiple clinical settings, including for the estimation of the likelihood of disease-free survival and determining possible platinum sensitivity. IHC evaluation of PDG2 was an independent marker of a good prognosis, thereby contributing to our understanding of a mechanism of tumour regulation. Although a number of challenges remain, the incorporation of properly validated biomarkers into clinical practice holds great potential for the improvement of HGSOC treatment.

Acknowledgements

The authors would like to thank Dr Corrado D'Arrigo and Dr Sarah Wedden (both Poundbury Cancer Institute, Dorset, England) for critical reading of the manuscript and all members from Poundbury Cancer Institute, for their valuable technical assistance on protein analysis.

Funding

Funding for this project was provided by the Foundation for Research Support of the State of São Paulo and Coordination for the Improvement of Higher Educational Personnel.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Research and design: MRA, NSDA, LDB, AABADC, FAS and RMR. Conducted the research: MRA, NSDA and FIDBS. Development of methodology: MRA, NSDA, FAM, FIDBS and KCC. Acquisition of patient data: AABADC and GB. IHC analysis and statistical analysis: MRA, LDB, AABADC. Manuscript revision in terms of intellectual content: AABADC and RMR. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Regional Ethics Committee at A.C. Camargo Cancer Center (São Paulo, Brazil; 1863/14).

Patient consent for publication

A consent form declaring that the patient is aware of data publication was applied, evaluated and approved by the Regional Ethics Committee at A.C. Camargo Cancer Center in 2014 (São Paulo, Brazil; 1863/14).

Competing interests

The authors declare that they have no competing interests.

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