HSPA1A, HSPA1L and TRAP1 heat shock genes may be associated with prognosis in ovarian epithelial cancer

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Abstract. Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy, with the presence of chemo-resistance contributing to the poor prognosis. Heat Shock Proteins (HSPs) genes are activated in response to patho-physiological stress and serve a role in a variety of stages in carcinogenesis, acting primarily as anti-apoptotic agents and in chemotherapy resistance in a variety of tumor types. The current study evaluated the HSP gene expression profile in women with ovarian cancer (OC) and their correlation with clinical and pathological aspects of patients with OC. A total of 51 patients included in the current study were divided into four groups: Primary Epithelial Ovarian Cancer (EOC; n=14), metastatic EOC (n=11), ovarian serous cystadenoma (n=7) and no evidence of ovarian malignancy or control groups (n=19). RNA extraction and reverse transcription-quantitative (RT-q) PCR was then performed on the samples obtained. RT-qPCR was performed to compare TNF receptor associated protein 1 (*TRAP1*), heat shock protein family (*HSP*) *HSPB1*, *HSPD1*, *HSPA1A* and *HSPA1L* expression in primary and metastatic EOCs. *TRAP1, HSPB1, HSPD1, HSPA1A* and *HSPA1L* gene expression did not differ among groups. *HSPA1A, HSPA1L* and *TRAP1* were revealed to be underexpressed in the primary and metastatic EOC groups, with *HSPA1L* exhibiting the lowest expression. *TRAP1* expression was higher in tumors at stages I/II compared with those at stages III/IV. No correlation was exhibited between HSP expression and age, menarche, menopause, parity, period after menopause initiation, cytoreduction, CA-125 or overall and disease-free survival. *HSPA1A* was negatively correlated with the risk of mortality from OC. The results indicated that the downregulation of *HSPA1A, HSPA1L* and *TRAP1* could be associated with the clinical prognostic features of women with EOC.

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Introduction

Epithelial ovarian cancer (EOC) causes around 125,000 deaths globally per year (1). Approximately 70% of women with ovarian cancer (OC) are diagnosed with locally advanced or metastatic disease (stages III/IV), of whom only ~30% will survive more than 5 years. By contrast, women diagnosed with earlier stage (stage I) disease have a 5-year survival rate >90%. Unfortunately, signs and symptoms of OC are usually absent or too subtle to be easily detected in the early stages of the disease. Despite of high initial response rates to chemotherapy, approximately 80% of women with advanced OC relapse within 2 years after initial drug treatment (2,3).

The standard treatment for EOC involves maximal cyto-reductive surgery followed by platinum and taxane-based chemotherapy. At first, most patients with advanced stage (III/IV) EOC respond well to surgery and chemo-therapy; however, within two years after initial treatment, cancer frequently relapses with a drug-resistant phenotype and most patients die of the disease (4). Age and disease staging at diagnosis, tumor histology, and performance status (PS) are the best known prognostic factors (5), albeit limited by our restricted understanding of EOC's biology and complicated by disease heterogeneity.

Currently, there is a growing interest in finding specific molecular markers that could function both in the prognosis of the disease and the patient's response to chemotherapy. Good candidates include heat shock proteins (HSPs) because of their role in facilitating malignant transformation, tumor progression, and tumor survival (6,7). These evolutionarily conserved proteins are classified according to their molecular weight and, in mammalian cells, are grouped into six main classes: HSP27, HSP40, HSP60, HSP70, HSP90, and HSP110 (7). The contribution of HSPs to tumorigenesis can be attributed to their activities governing folding/unfolding, turn-over, and transport of client proteins as well as assembly of multiprotein

complexes. As a result, various crucial and clinically important cell responses are vitally influenced and modulated by HSPs, e.g., cell growth, apoptosis, metastasis, and treatment resistance (6,7).

Although the existing data for HSP's function in OC progression and drug resistance is appealing, it is still limited and conflicting at times. For instance, the cytosolic HSPB1(HSP27), HSP70 (HSPA1A, HSPA1L), and HSP90(TRAP1) as well as the mitochondrial HSP60 proteins and the tumor necrosis factor receptor-associated protein 1 (TRAP-1) have all been shown to be induced by drug treatment and frequently associated with cross-resistance to anticancer compounds of different classes in ovarian and other cancer types (6-10). Moreover, the levels of circulating HSP27 protein were decreased after chemotherapy treatment in metastatic OC patients (11). Thus, drug-mediated regulation of HSPs in OC may follow differentially controlled stress signaling pathways. Due to our limited understanding of HSP's role in OC biology, studies elucidating their potential to help the prognostic evaluation of patients and the therapeutic strategy upon relapse after platinum-based chemotherapy are warranted.

In the present study, we correlated the expression of *TRAP1, HSPB1, HSPA1, HSPAl,* and *HSPD1* genes and the clinical and pathological aspects of patients with OC. To this end, we compared the expression of these genes in the primary and metastatic ovarian tumor and investigated the relationship between the observed expression profile with other known prognostic factors and with the patients' response to chemotherapy and relapse-free survival.

Materials and methods

Ethics. This study was approved by the Research Ethics Committee of Vera Cruz Hospital (Belo Horizonte, Minas Gerais, Brazil), under the protocol CAAE: 01242212.2.0000.5135. All participants voluntarily signed an informed consent form.

Patients and tumor tissue samples. We collected ovarian tissue from 51 women divided into four groups: Primary Epithelial Ovarian Cancer EOC (n=14), metastatic EOC (n=11), ovarian serous cystadenoma (n=7) and normal ovary (n=19). The patients were recruited to our study using convenience sampling and they did not match any of the following exclusion criteria: Previously treated with chemotherapy and/or radiotherapy; HIV positive; presenting any infectious process diagnosed or not during laparotomy; present or previous history of other malignant neoplasms; using or with a previous history of use of immunosuppressives, systemic corticosteroids or non-steroidal anti-inflammatory drugs in the three months prior to the study. All cases were reevaluated blindly by a senior consultant subspecialized in gynecologic pathology and a representative portion of each tumor containing >80% tumor cells were selected for storage until analysis. Clinical and pathologic information documented at the time of surgery included disease stage, tumor grade and histotype, residual tumor size and debulking success.

In the EOC patients, samples were collected from primary tumors and of metastatic tumors, when extra pelvic disease above 1 cm was observed. Tumor staging was performed according to the FIGO recommendations (12). Normal ovarian epithelial tissue samples were taken from postmenopausal women who required a bilateral oophorectomy. After excision, the samples were immediately frozen in liquid nitrogen and stored at -80˚C until use.

RNA extraction, cDNA synthesis and gene expression analysis. Total RNA was extracted from 50 to 100 mg of each ovarian tumor sample using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The RNA yield and A260/280 ratio were determined by a NanovueTM Plus Spectrophotometer (GE Healthcare Biosciences). RNA integrity and quality were characterized through 1% agarose gel electrophoresis. Subsequently, the samples were treated with RNAse-Free DNAse Set® (Qiagen) to remove possible traces of genomic DNA.

cDNAs were synthesized using M-MLV Reverse transcriptase (Promega Corporation) according to the manufacturer's recommendations and were subjected to RT-qPCR using TaqMan® Universal PCR master mix and inventoried TaqMan® Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's recommendation. Taqman assays were selected for each target gene: *TRAP1* (Hs00212476_m1), *HSPB1* (Hs00356629_g1), *HSPA1A* (Hs00359163_s1), *HSPA1L* (Hs00271466_s1), *HSPD1* (Hs01036753_g1) and for *TBP* (Hs00427620_m1) used as endogenous control. A sample without a template was included as a control in each assay. Each 40-cycle reaction was performed in duplicate using a Step OnePlus detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Two technical replicates were adopted for each sample. Relative gene expression was determined using the $2^{-\Delta\Delta Cq}$ method (13).

Gene functional and Network pathway analysis. The differentially expressed genes determined using the $2^{-\Delta\Delta Cq}$ method and for the pathway analysis of gene-associated proteins, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was investigated by using STRING database, version 10.5 (14). STRING was also used to evaluate protein-protein interactions (PPI) among the associated genes.

Statistics. Student's t-test and ANOVA were used to compare gene expression and qualitative variables (15). To detect correlation between the genes and to compare their expression with the quantitative variables, we used Pearson's correlation and Spearman's correlation (16), respectively. To compare disease free time curves and survival curves with gene expression, the log-rank test (17) was used. It is worth mentioning that the gene expressions were recoded as greater than 1, less than 1, or equal to 1. To analyze the factors influencing survival and the disease-free interval, a univariate analysis was performed using the Cox Regression Model and the Risk Ratio was computed (17). Different from logistic regression, the Cox model has the advantage of including the effect of time up to the death and relapse besides allowing the interpretation through the risk ratio and not the odds ratio. The probability of survival and significance were calculated using the Kaplan-Meier method (18). All statistical analyzes were performed using the statistical software package R (version 3.4.1) (http://www.r-project.org/), using stats package (19) to quantitative variables and survival package

Table I. General characteristics of patients.

Variables	Cystadenoma mean ± standard error	Primary EOC mean ± standard error	Metastatic EOC mean ± standard error	Normal ovary mean ± standard error	P-value[a]
Age (years)	50.00±16.54	57.93±10.54	59.55±10.76	47.68±8.33	0.017
Menarche	12.57±1.13	12.79±1.37	13.10±1.45	-	-
Parity (births)	2.14±3.08	2.00±1.24	2.10±1.20	-	-
Period after Menopause	6.86±11.19	8.62±9.03	10.13±9.03	-	-

[a]Kruskal-Wallis. EOC, epithelial ovarian carcinoma.

Table II. Clinicopathologic characteristics in ovarian sample.

Variables	Cystadenoma N (%)	Primary EOC N (%)	Metastatic EOC N (%)	Normal ovary N (%)	P-value[a]
Stage					
I	0 (0.0)	5 (35.7)	2 (20.0)	-	-
III	0 (0.0)	6 (42.9)	6 (60.0)	-	
IV	0 (0.0)	3 (21.4)	2 (20.0)	-	
Menopause					
No	4 (57.1)	3 (21.4)	2 (20.0)	17 (89.5)	<0.001
Yes	3 (42.9)	11 (78.6)	8 (80.0)	2 (10.5)	
Ascites					
No	7 (100.0)	4 (30.8)	3 (37.5)	-	-
Yes	0 (0.0)	9 (69.2)	5 (62.5)	-	
Tumor differentiation grade					
G2	0 (0.0)	5 (35.7)	4 (40.0)	-	-
G3	0 (0.0)	9 (64.3)	6 (60.0)	-	
CA-125					
<35 U/ml	3 (42.9)	4 (28.6)	1 (9.10)	-	-
>35 U/ml	4 (57.1)	10 (71.4)	10 (90.9)	-	

[a]Fisher exact test. EOC, epithelial ovarian carcinoma.

to analyze the survival rates (20). P<0.05 was considered to indicate a statistically significant difference.

Results

The general characteristics of the patients are shown in Table I. The parity was 2.07 births with a range between 0 and 8 deliveries. The clinicopathologic characteristics of the tumor samples are shown in Table II. The stage was I in 7 patients (29.17%) and III/IV in 17 patients in the EOC group (70.83%). All samples were identified as high-grade serous carcinoma by histopathological evaluation.

TRAP1, HSPA1A, HSPA1L, HSPD1 and *HSPB1* genes showed differential expression between tumor samples of the EOC group and samples from the cystadenoma, primary and metastatic EOC samples. Although, no significantly differ among the groups (P>0.050; Fig. 1). When the groups were compared singly, *HSPA1A, HSPA1L* and *TRAP1* were significantly under-expressed in the primary and metastatic EOC groups in comparison to the expression profile presented in normal ovarian tissues, with *HSPA1L* showing the lowest expression in both carcinoma groups (Fig. 2).

There was no correlation between the expression levels of the analyzed genes and age, menarche, parity or period after menopause initiation as well as between the seric levels of the CA125 tumor marker and the expression of the HSP genes analyzed (Table III).

A comparison between the expression profile of the HSP genes and the OC staging showed that *TRAP1* expression was significantly greater in tumors at stage I than in tumors at stages III and IV of EOC patients (P=0.040; Table III).

There was no correlation between cytoreduction and the expression of the HSP genes analyzed herein (Table III). There were no significant differences (P=0.05) between the

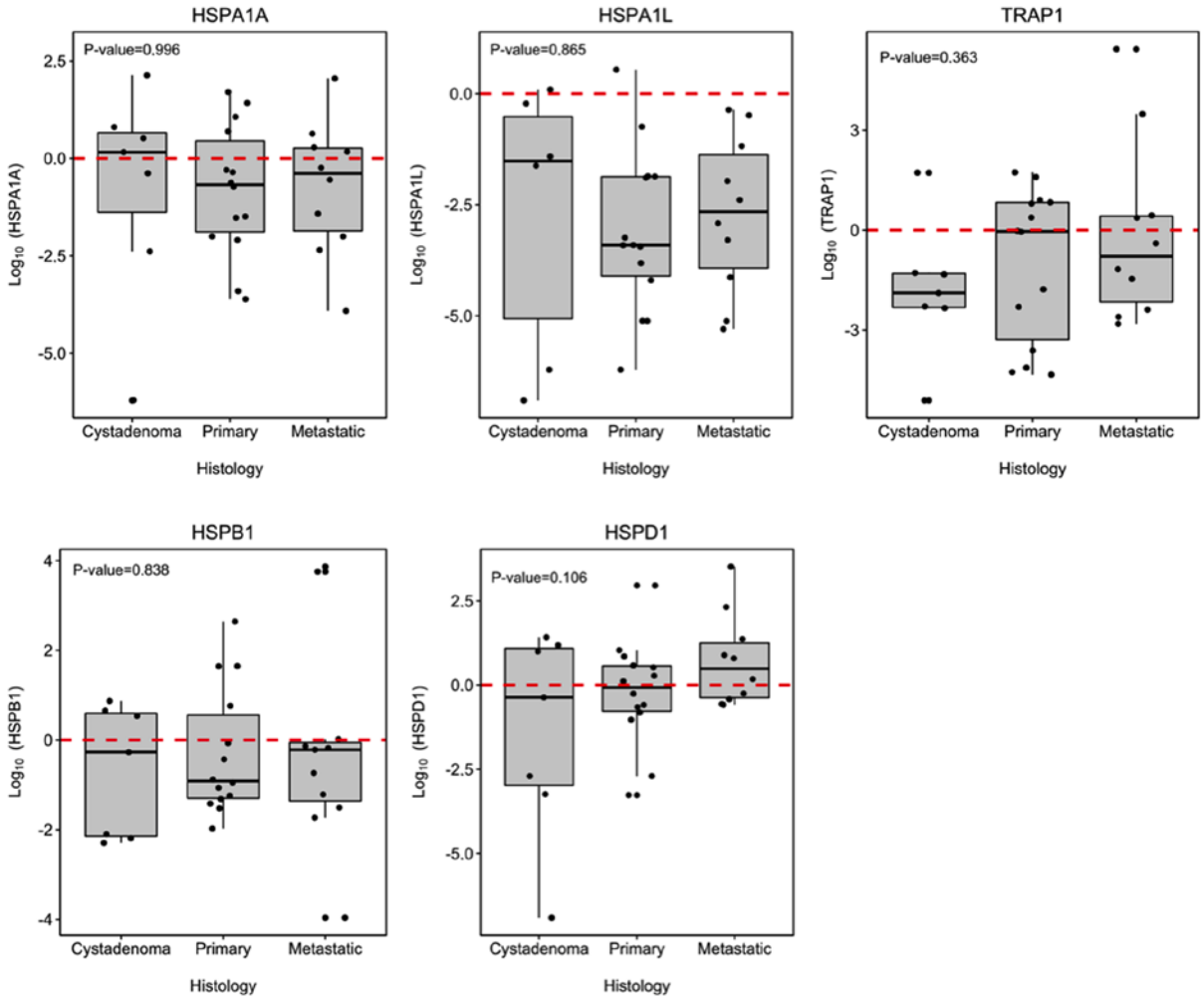

Figure 1. The association between *TRAP1*, *HSPD1*, *HSPB1*, *HSPA1L* and *HSPA1A* expression in ovarian tumors. The values represent *TRAP1*, *HSPD1*, *HSPB1*, *HSPA1L* and *HSPA1A* expression. The horizontal line indicates the median expression ratio, and the box plots demonstrate the interquartile range (25-75%). The 10 to 90th percentile ranges are also presented. The differences between groups were evaluated using a Man-Whitney U test and a Wilcoxon test. TRAP1, TNF receptor associated protein 1; HSP, heat shock protein family.

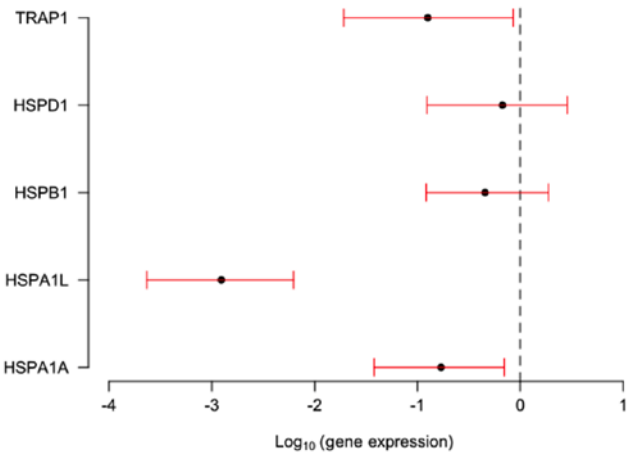

Figure 2. Comparison of the expression of the *TRAP1*, *HSPD1*, *HSP1*, *HSPA1* and *HSPA1A* genes in patients diagnosed with primary and metastatic EOC compared with patients with normal ovaries. Patients with normal ovary were considered as a control group (value of the expression equals 1, logarithm equals 0). The confidence intervals (red stems) of the genes intersecting the dashed line do not differ from each other. Intervals with confidence below the dashed line (less than 0) indicate that the gene is underexpressed. TRAP1, TNF receptor associated protein 1; HSP, heat shock protein family; EOC, epithelial ovarian carcinoma.

expression of the HSP genes evaluated and overall survival (OS) or disease-free survival (DFS) (Figs. 3 and 4). However, the gene expression analysis in relation to OS suggested influence of *HSPA1A* expression levels on the risk of dying of EOC (P=0.048). An increase of one unit in the gene log decreased the risk of dying by 0.73 times [0.53; 0.99] (Table III).

In silico network protein analysis made on STRING database revealed the protein-protein interactions between the proteins codified by the genes analyzed by us (Fig. 5).

Discussion

EOC is a very heterogeneous disease and the most lethal gynecological neoplasia (21). Despite extensive effort, EOC continues to be a poorly understood disease and patients survival rates remain low. Therefore, new strategies for early diagnosis, prognostic markers for clinical assessment and a better understanding of the mechanisms related to ovarian carcinogenesis are of extreme importance in order to obtain better outcomes for the affected patients. In this study, we investigated whether a gene signature among patients with and without EOC could be identified. To this end, we evaluated

Table III. Association between clinicopathologic characteristics of EOC patients and *TRAP1, HSPD1, HSPB1, HSPA1L* and *HSPA1A* gene expression profile.

Clinicopathologic characteristics	HSP gene expression profile correlation				
	HSPA1A	*HSPA1L*	*HSPB1*	*HSPD1*	*TRAP1*
Histopathology	0.996	0.865	0.838	0.106	0.363
Age (years)	0.797	0.309	0.723	0.287	0.451
Menarche	0.782	0.713	0.68	0.554	0.351
Parity (births)	0.119	0.061	0.852	0.152	0.594
Period after Menopause	0.804	0.643	0.486	0.632	0.409
CA-125	0.222	0.806	0.539	0.842	0.315
Stage	0.962	0.327	0.075	0.193	0.040
Menopause	0.927	0.664	0.786	0.600	0.492
Ascites	0.562	0.573	0.585	0.174	0.798
Tumor differentiation grade	0.397	0.305	0.035	0.080	0.163
Cytoreduction	0.797	0.772	0.239	0.824	0.422
Risk of dying	0.73	1.09	0.92	1.02	0.94
(P-value)	(0.048)	(0.491)	(0.527)	(0.892)	(0.511)

TRAP1, TNF receptor associated protein 1; HSP, heat shock protein family; EOC, epithelial ovarian carcinoma.

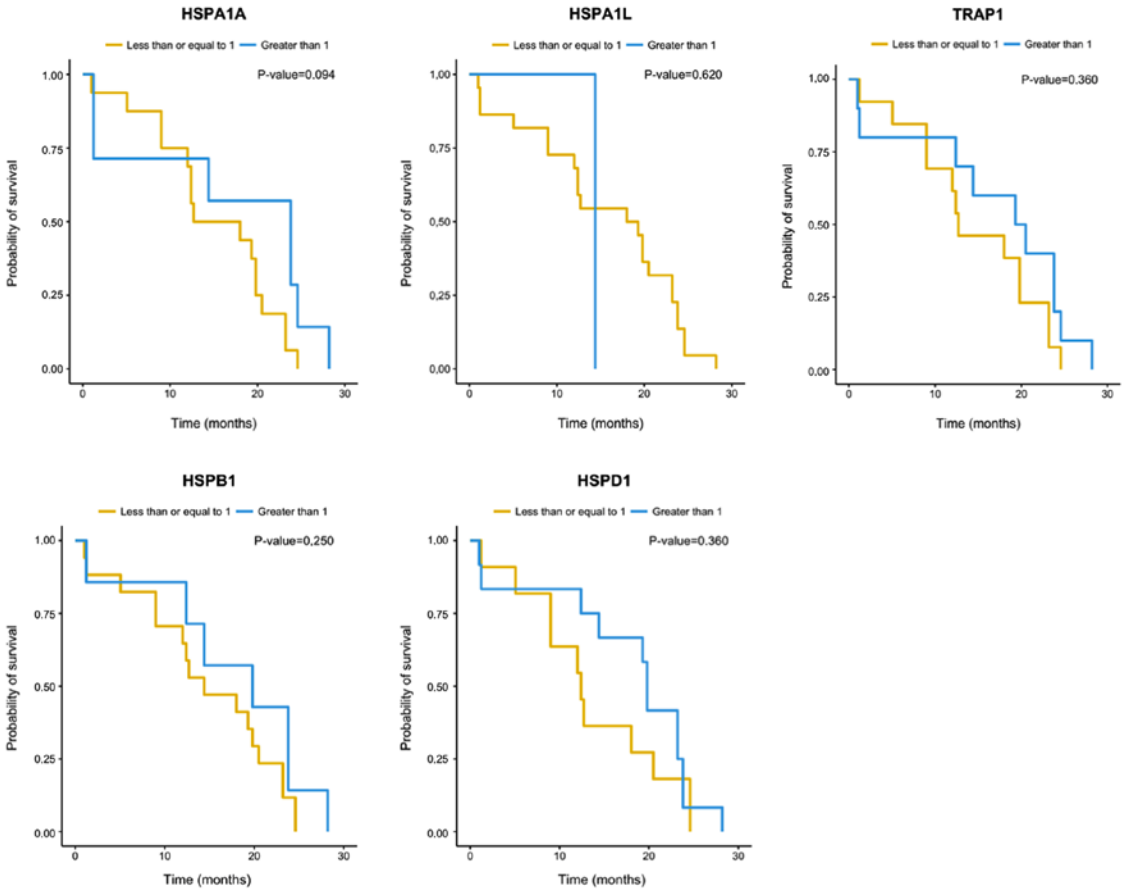

Figure 3. Kaplan-Meier analysis of overall survival among women with primary and metastatic EOC according to their *TRAP1, HSPD1, HSPB1, HSP*A1L and *HSPA1A* gene expression. TRAP1, TNF receptor associated protein 1; HSP, heat shock protein family; EOC, epithelial ovarian carcinoma.

the level of expression of the genes *TRAP1, HSPD1, HSPB1, HSPA1A*, and *HSPA1L* in tumor samples obtained from patients with cystadenoma, primary and metastatic EOC in relation to baseline expression of these genes in normal ovary

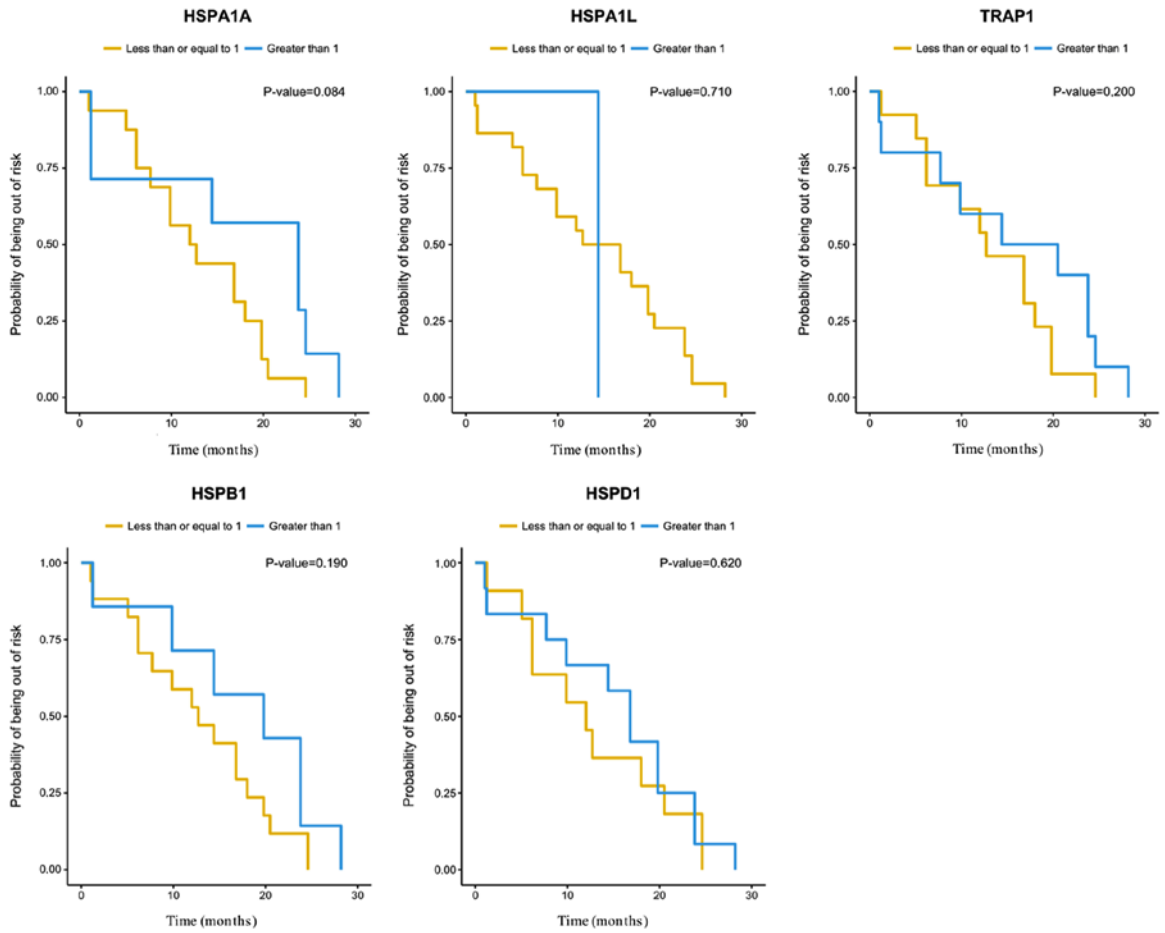

Figure 4. Kaplan-Meier analysis of disease-free survival among women with primary and metastatic EOC according to their *TRAP1, HSPD1, HSPB1, HSPA1L* and *HSPA1A* gene expression. TRAP1, TNF receptor associated protein 1; HSP, heat shock protein family; EOC, epithelial ovarian carcinoma.

(NO) tissues. Therefore, expression of each gene in NO was assigned an arbitrary quantity of '1' and their expression in the tumor samples were expressed in terms of their fold difference to NO (21). We found that these five genes were differentially expressed between the groups, but the prediction of EOC metastasis with gene expression profiling was not better than chance alone. The comparison between the expression levels of the studied genes in the tumor groups with the NO group showed that *HSPA1A, HSPA1L* and *TRAP1* were significantly under-expressed in the EOC groups.

The under-expression of HSP70 isoforms was previously observed in OC (22). According to these authors, the genes *HSPA1A* and *HSPA1L* reside on a particularly vulnerable CpG island, which is subject to methylation and boosts the immune response. In addition, the copy number variation (CNV) of the HSP genes described for different tumors may also explain the under-expression of the observed *HSPA1A, HSPA1L* and T*RAP1* genes in our study. *TRAP1* expression is correlated with the copy number, suggesting this could be one of the driving mechanisms for the loss of *TRAP1* expression in OC (23).

Several gene expression studies identifying molecular markers related to cancer progression have been published. Overall, there is a considerable overlap between previous studies and our study in terms of differentially expressed genes between normal and tumor tissues. Furthermore, all studies demonstrate the great diversity of tumor pathobiology, a feature that makes cancer a difficult disease to treat effectively (24-26).

TRAP1, HSPD1, HSPB1, HSPA1A, and *HSPA1L* belong a stress or HSPs family of highly conserved genes that are expressed in response to a wide variety of physiological and environmental insults in order to maintain cellular homeostasis or to contribute to cell survival to lethal conditions. The stresses involving HSPs include such as hypoxia, exposure to UV light and chemicals, viral agents, nutritional deficiencies (e.g., glucose deprivation), surgical, emotional and mechanical stress, among other stresses (27-30). Beyond that, biological processes of proteins among HSP associated genes analyzed by the Gene functional and Network pathway analysis performed herein revealed their function in chaperone mediated protein folding.

TRAP1 encodes a mitochondrial chaperone protein that is a member of the heat shock family 90 (HSP90). The protein has ATPase activity and interacts with tumor necrosis factor type I (31). Interestingly, alternate splicing results in multiple transcript variants (32) and other study suggested that TRAP1 has an oncogenic role in a variety of cancer types (33). In colorectal carcinoma, increased expression of TRAP1 was correlated with increased lymph node involvement, more advanced stages of the disease, and reduction in overall survival. TRAP1 is currently a marker predicting worse outcomes in colorectal cancer (34). However, low levels of TRAP1 has been

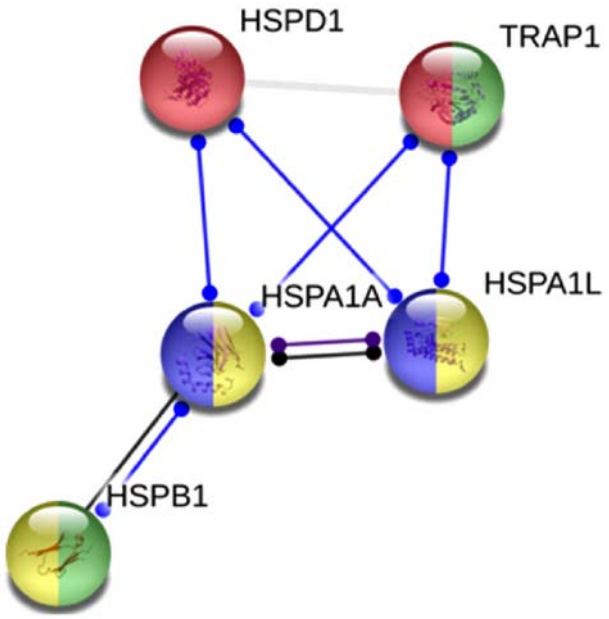

Figure 5. Gene functional and Network pathway analysis of T*RAP1, HSPD1, HSPB1, HSPA1L* and *HSPA1A*. The Gene functional and Network pathway analysis evaluated in silico by STRING database. The circles colors indicate the HSP functions in network. Chaperone mediated protein folding, negative regulated of oxidative stress-induced intrinsic apoptotic signaling pathway (green), MAPK signaling pathway (yellow), and spliceossoma (purple). TRAP1, TNF receptor associated protein 1; HSP, heat shock protein family.

related to high tumor grade, more advanced stage and resistance to platinum in OC (35). In OC cells lines and tissues, TRAP1 was shown to be associated with a metabolic shift, ultimately causing the onset of resistance to cisplatin-based chemotherapy (36). Furthermore, in OC clinical samples, *TRAP1* is often deleted in high-grade serous OC patients and it is correlated directly with epithelial-mesenchymal transition, which is an important determinant of the invasive potential of tumor cells (23). Therefore, TRAP1 downregulation is linked to tumor progression in OC patients. Similarly, *TRAP1* was under-expressed in the EOC group compared with the NO group and higher expression was showed in tumors at stages I/II than at III/IV (P=0.040), a finding that could be correlate with a negative impact on the response to chemotherapy and survival of patients with OC.

The *HSPA1A, HSPA1L, HSPD1*, and *HSPB1* genes are expressed either constitutively or regulated inductively. High molecular weight HSPs are ATP-dependent chaperones (*HSPA1A, HSPA1L, HSPD1*), whereas small HSPs act in an ATP-independent fashion (*HSPB1*). As molecular chaperones, the function of HSPs is to regulate protein folding, transport, translocation and assembly, particularly to refold misfolded proteins or assist their elimination (27,30). The literature relates the overexpression of these genes as a possible marker of worse prognosis in other tumor types (37). It is known that in cancer there is a need for ambiguous signal transduction, hence there are greater demand for chaperones. The phenomenon is probably linked to the drastic changes in protein homeostasis caused by the accumulation of mutated proteins in cancer cells (27).

In our study, *HSPA1A* was the gene that presented the lowest level of expression in relation to the NO group. In addition, it showed a significant influence on the overall survival of patients with EOC, who showed a decrease in their risk of death by 0.73 times for every increase in one unit in *HSPA1A* expression, suggesting a protective role for this gene. This result highlights the potential of this gene as a possible genetic marker to assist the clinical evaluation of the prognosis of the disease.

The association between *HSPB1* expression and high-grade OC primary tumors and metastases was described previously (38) and similar results were already observed in cell lines (39). The immune response to HSPB1 is also increased in women with OC and other gynecological tumors and some studies suggested the use of anti-HSP27 antibody concentrations for early diagnosis of relapse or disease progression (40). The association of HSPB1 with early disease staging and longer survival of patients with OC, most studies suggest an association between HSPB1 overexpression and worse prognosis (41). There appears to be a co-expression of HSPB1 or a positive correlation between HSPB1 expression and resistance to chemotherapy and expression of *MDR1* (gene for resistance to multiple drugs) (42). Thus, there is evidence that the overexpression and activity of HSP27 is associated with increased carcinogenesis, metastatic potential, and resistance to chemotherapy. However, in our series the expression of *HSPB1* was not significantly different in the EOC group in comparison to the control group.

This study has some limitations which have to be considered. The small number of patients and controls do not allow us to draw any meaningful conclusions regarding the relation between gene expression and anatomic site or clinicopathologic parameters. It is worth mentioning that, differently from tumors studies in other sites, the low prevalence of ovarian tumors, along with the ethical and biological determinants for control group selection imposes a limiting factor of patient numbers in EOC cohorts. So, our study was led with convenience samples, also known as availability sampling, a specific type of nonprobability sampling method that relies on data collection from a population who are conveniently available to participate in study. Because of that, it observed the unbalanced number of samples of each group. Furthermore, the lack of immunohistochemistry to confirm the expressions of HSP genes could be considered other limitation to our study. However, it was designed to evaluate gene expression by tracking messenger RNA using RT-qPCR, due access to standardized protocols and automation ensures an accurate performance and fast turn-around. Our findings highlight the importance of understanding the role of HSP genes in the ovarian carcinogenesis process and future investigations should be performed cloning HSP genes into OC cell lines or using CRISPR gene editing to verify if chemoresistance and others prognosis features can be altered in OC by HSPs gene expression and confirm our results.

We can hypothesis that *HSPA1A*, *HSPA1L* and *TRAP1* downregulation seems to enhance the ability of the cancer cells to die in a range of lethal conditions. Further studies with a larger number of patients and longer follow-up are necessary to assess the accuracy of the prognostic impact of these results.

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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

Regarding the authorship of the manuscript, AWP, SAL, BLC, GNG and SLM gave individual contribution in the concept and design/analysis and interpretation of data, drafting the manuscript or revised it critically for important intellectual content and final approval. AWP and SAL were responsible for patient recruitment. BLC, GNG and SLM were responsible for performing the experimental assays.

Ethics approval and consent to participate

The current study was approved by the Research Ethics Committee of Vera Cruz Hospital, Belo Horizonte, Brazil, under the protocol CAAE: 01242212.2.0000.5135. Informed consent forms were obtained when the patients were accepted for the study by the hospital.

Patient consent for publication

Consent for publication was obtained from all participants of the current study.

Competing interests

The authors declare that they have no competing interests.

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