Am J Cancer Res 2021;11(4):1754-1769
www.ajcr.us /ISSN:2156-6976/ajcr0130053

Original Article

The shifting landscape of genetic alterations separating endometriosis and ovarian endometrioid carcinoma

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Received January 13, 2021; Accepted February 14, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: Ovarian cancer is one of the most common cancers worldwide, and is associated with a prior diagnosis of endometriosis in several cases. Our aim was to correlate genetic and methylation profile of ovarian endometrioid ovarian cancer and endometriosis patients. We evaluated the genetic profile of 50 ovarian endometriosis and 20 ovarian endometrioid carcinoma samples using next generation sequencing technology. In addition, the DNA methylation profile was evaluated for both cohorts of patients. We observed several mutated genes that were common for both types of patients, but we also identified mutated genes that were characteristic for each group: *JAK3, KRAS* and *RB1* for endometriosis; and *ATM, BRAF, CDH1, EGFR, NRAS, RET* and *SMO* for ovarian endometrioid cancer. Also we idenfied genes that are highly methylated only in endometriosis samples (*PYCARD, RARB, RB1, IL2, CFTR, CD44* and CDH13) and MLH3 gene was methylated only in endometrioid ovarian carcinoma samples. Also, *BRCA1, CADM1, PAX6* and *PAH* genes are mainly methylated in endometrioid ovarian carcinoma patients. We identified a correlation for the cancer group between tumor stage, copy number aberrations and the presence of metastases; more specifically, the presence of *BRCA1* pathogenic variants was correlated with tumor differentiation degree, *TP53* variants and copy number aberrations. This study was able to demonstrate the presence of similar pathways being altered in both endometriosis and ovarian endometrioid carcinoma, which could mean that a diagnosis of endometriosis could be an early marker for cancer diagnosis. In addition, we showed that *GATA2* hypomethylation, *ATM* hypermethylation, *CREM* hypomethylation, higher tumor differentiation degree or higher tumor stage is associated with a poor prognosis in patients with ovarian endometrioid carcinoma.

Keywords: Endometriosis, ovarian endometrioid carcinoma, next-generation sequencing, methylation profile, *BRCA1/2*

Introduction

Endometriosis is a benign disease accompanied by chronic inflammation and pain. Ten to fifteen percent of women at reproductive age are affected by endometriosis. To date, no cure has been found for this disease and, in some cases, endometriosis can be transformed in endometrial cancer [1, 2]. In addition, a preexisting diagnosis of ovarian cists or endometriosis has been shown to be associated with a higher risk of developing ovarian cancer [3]. Endometriosis is clinically defined as the presence of endometrial-like tissue, including glands and stroma, outside of the uterus in anatomically ectopic locations. It is an estrogen dependent disease and is mainly localized in the peritoneum, uterus and ovaries [4]. Association of endometriosis and ovarian cancer was demonstrated by several studies, and they

Table 1. Clinical data of the ovarian endometrioid carcinoma patients included in the study

		No of patients-ovarian endometrioid carcinoma n=20 (%)
Age	Median	59.4
	range	44-78
Differentiation grade	G1	1 (5%)
	G2	6 (30%)
	G3	6 (30%)
	Unavailable	3 (15%)
Tumor stage	T1	7 (35%)
	T2	3 (15%)
	T3	7 (35%)
	NA	3 (15%)
Lymph nodes	N0	3 (15%)
	N1	1 (5%)
	NX	13 (65%)
	Unavailable	3 (15%)
Metastasis		13 (65%)
Deceased		8 (40%)
Treatment	Paclitaxel + carboplatin	13 (65%)
	Doxorubicina + cisplatin	1 (5%)
	Carboplatin	1 (5%)
	unavailable	5 (25%)

have demonstrated that the two main subtypes of ovarian carcinoma that can be developed from endometriosis are endometrioid and clear cell [5-8].

Ovarian cancer is the seventh diagnosed type of cancer and the fifth cause of death by cancer in Europe [9]. Worldwide, ovarian cancer is the eighth most diagnosed type of cancer and the eighth cause of death by cancer [10]. Ovarian endometrioid carcinoma is diagnosed in 18-20% of women affected by ovarian cancer and it has been shown that this particular ovarian cancer subtype is correlated with a preexisting endometriosis [11, 12]. Epithelial ovarian cancer is classified in several subtypes, with ovarian serous carcinoma being the most common (60%), followed by ovarian endometrioid carcinoma (10-20%), ovarian clear cell carcinoma (5%) and mucinous carcinoma (<5%) [13]. Even though most ovarian cancers are sporadic, 5-10% of all ovarian cancer patients are clinically diagnosed with an inherited disease. The inherited ovarian cancers are generally correlated with germline pathogenic variants, located in the *BRCA1* and *BRCA2* genes [14]. Ovarian cancer and each of its subtypes are associated with alterations in different genes, either variants or expression deregulations [15]. However, it is important to note that these alterations do not represent all the factors needed for cancer progression and metastasis. There are also different epigenetic events that contribute to the development and progression of ovarian cancer, making them desirable for the targeted treatment of this disease [16]. Another important aspect is the reversibility of the epigenetic alterations. One of the most studied epigenetic alterations, which showed promising results in both diagnosis and treatment of ovarian cancer, is DNA methylation [17].

In the present study, we tried to correlate the genetic profile of endometriosis patients with the ovarian endometrioid carcinoma ones, by investigating their *BRCA1/2* status, DNA methylation and gene variant profiles identified in a specific subset of genes correlated with cancer.

Materials and methods

Patient cohorts

This study included 50 patients with ovarian endometriosis and 20 patients with ovarian endometrioid carcinoma. The patients were diagnosed and treated at the Oncology Institute "Prof. Dr. Ion Chiricuta" Cluj-Napoca between the years 2013 and 2015. The patients with endometriosis had a mean age of 35.8, with an age range of 22-51 years old. The clinical data of the patients with endometrioid ovarian carcinoma included in the study are presented in **Table 1**. All patients were provided with comprehensive details about the study and signed an informed consent. The study was approved by the Ethical Committee of the "Iuliu Hatieganu" University of Medicine and Pharmacy-Cluj-Napoca no. 74/16.02.2015, and by the Ethical Committee of The Oncology Institute "Prof. Dr. Ion Chiricuta" no. 64/10.03.2017, this being a retrospective study.

All the pathological samples were reviewed by another pathologist and macro-dissection was done on each sample.

DNA extraction

DNA was extracted from formalin fixed paraffin embedded tissue (FFPE) and fresh frozen tissue using the Purelink Genomic DNA mini kit (Invitrogen), using the manufacturer's protocol. The concentration and quality of DNA was evaluated using NanoDrop (ThermoFischer). The concentrations were between 28.5-801.1 ng/μl, with a 260/280 ratio between 1.62 and 1.82, and the 260/230 ratio between 1.26 and 2.27.

Next generation sequencing (NGS)

Next generation sequencing experiments were done using a quantity of 10 ng of DNA for each sample. We used two primer panels, namely the Ion Ampliseq cancer panel pool and Ampliseq Community *BRCA 1_BRCA2* primer kit from Life Technologies. The Ion Ampliseq cancer panel contains primer pairs for 190 amplicons of 46 oncogenes and tumor suppressor genes and covers 739 COSMIC variants in 604 loci, providing 97% coverage. The Ion Ampliseq Community *BRCA1_BRCA2* primer kit consists of primer pairs for 167 amplicons that cover the coding regions of both *BRCA1* and *BRCA2* genes. The sequencing libraries and the actual sequencing process were conducted according to the protocol described in our previous paper [18].

NGS data analysis

For signal processing, base calling and sequence alignment, we used the Torrent Suite V4.4 software (Life Technologies), and sequences were aligned to the Human Genome Build 19 (hg19). We used the variant Caller 4.4.0.6 plugin for detecting variants, with Target Regions settings specific for the two AmpliSeq panels. For annotations, we transferred the VCF files generated for each sample to the Ion Reporter 4.6 software, and used the following filters: p value ≤ 0.05, coverage ≥ 500 and frequency ≥ 10.

Mutation validation

For the validation of variants, we used TaqMan SNP Genotyping assays from Life Technologies. For variants from ovarian endometrioid carcinoma cohort we used the following assay: AKT1 rs3730358, assay ID C____193157_10, KDR c.1416T>A, rs1870377, assay ID C__11895315_20, and for variants from the endometriosis cohort we used the following assays: AKT1 rs3730358, assay ID C____193157_10, KDR c.1416T>A, rs1870377, assay ID C__11895315_20 and KIT rs3822214, custom assay. The assays were used in a real-time PCR reaction together with the TaqMan Genotyping Master Mix (Life Technologies); we used the protocol provided by the manufacturer as described in our previous work [18].

Evaluation of the methylation profile and copy number alterations

In order to evaluate the methylation profile and the copy number alterations in our samples, we used Salsa MLPA ME002 Tumor suppressor mix 2 probemix (MRC Holland). Primarily, we used 100-150 ng of DNA from each sample. The mixture was denaturated at 95°C for 5 minutes, and then hybridized with a mixture of MLPA buffer (1.5 μl) and Probe mix (1.5 μl) at 60°C for 20 h. After hybridization, 3 μl of Ligase Buffer A and 10 μl of nuclease free water were added and mixed thoroughly. 10 μl of this mixture was digested using the HhaI restriction enzyme (Promega). For the ligation reaction without digestion, we used 10 μl of mixture, 8.25 μl of nuclease free water, 1.5 μl of ligation buffer B and 0.25 μl of Ligase 65. For ligation reaction with digestion, we used 7.75 μl of nuclease free water, 1.5 μl of ligation buffer B, 0.25 μl of Ligase 65, and 0.5 μl of HhaI enzyme. Both reactions were incubated 30 min at 48°C and 5 min at 85°C. After this step, we amplified both reactions using a mixture of nuclease free water (3.75 μl), Salsa PCR Primer mix (1 μl) and Salsa Polymerase (0.25 μl). The PCR program consisted of 35 cycles: 95°C-30 sec, 60°C-30 sec, 72°C-60 sec, 1 cycle-72°C-20 min and 1 cycle 15°-∞. The products obtained were run on an ABI 310 capillary sequencing machine (Applied Biosystems) using the manufacturer's instructions. The data generated was analyzed using the Coffalyser software (MRC Holland) and Excel (Microsoft Office). The copy number alterations were obtained directly from the Coffalyser software. The methylation dosage ratio (Dm) was calculated using the formula: $Dm=(P_x/P_{ctr})_{dig}/(P_x/P_{ctr})_{undig}$, where P_x-peak area of a given probe, P_{ctr}-sum of the peak areas of the control probes, dig-represents the HhaI digested sam-

ple, undig-represents the undigested sample. Dm can be between 0-1, corresponding to the 1-100% of methylation DNA, and all samples that had a Dm ≤ 0.15 were excluded.

Methylation profile of ovarian cancer patients in TCGA databases

The curated TCGA data of ovarian cancer was downloaded from UCSC Xena database and the beta-values from genes of interest were selected, (https://xenabrowser.net/datapages/?dataset=TCGA.OV.sampleMap%2FHumanMethylation27&host=https%3A%2F%2Ftcga.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443). The dataset included 582 tumor tissue samples and 12 normal tissue samples. The samples were divided between normal and tumor samples, based on their barcode. The density plots were constructed in R, with the use of dplyr and ggplot2 packages. As grouping method, the type of sample was selected, with "dodge" position and alpha=0.4. For the statistical analysis, the *p* value based on the distribution of beta-values density calculated, with the help of Kolmogorov-Smirnov test. The resulted *p*-values were included on the plots. In order to better observe the methylation differentiation between normal and tumor samples, an arithmetic mean of beta-values from normal samples was calculated and the differentiation of percent methylation between this mean and each beta-value from tumor tissue was calculated based on a custom made function in R. All the values of percent data from tumor tissue, for all the selected genes were included in a horizontal violin plot using Graph Pad software.

Mutation profile of ovarian cancer patients in TCGA databases

The general processed mutation data of ovarian cancer was downloaded from UCSC Xena database (https://xenabrowser.net/datapages/?cohort=TCGA%20Ovarian%20Cancer%20(OV)&removeHub=notebook%3A). For base substitution, the reference base and alternative base of each SNP in the database were included in a separate column. Then the genes of interest were selected and included in a boxplot by using ggplot2 R package. In order to observe the frequency of mutations in ovarian cancer in the genes of interest, the Ovarian Serous Cystadenocarcinoma (TCGA, PanCancer Atlas) dataset was selected from cBioPortal database (https://www.cbioportal.org/study/summary?id=ov_tcga_pan_can_atlas_2018) dataset was used. This dataset includes 523 tumor samples of ovarian cancer. The full list of mutated genes was downloaded and only the genes of interest were selected. The percents of frequencies were converted in values from 0 to 1. The ggplot2 and ggpubr packages were used to draw the dot chart, with set ymax = maximum percent of mutation frequency per gene.

Statistical analysis

All statistical analysis was done in Graph Pad Prism v.6.0. Pearson test was used for determining statistical associations between: each variant; the variants and tumor grade; and lastly, tumor size and death. For survival analysis, Kaplan-Meier survival curves were implemented together with Cox analysis for evaluation of *p* value and log rank for hazard ratio. Gene ontology analysis was performed using the free online Panther DB program in which we uploaded the list of genes that contained the determined variants for each disease.

Results

Mutation identification-cancer panel

From the cancer panel, 85 different variants in 32 genes were identified for the ovarian endometrioid carcinoma patients, while 23 different variants in 17 genes were identified for the endometriosis patients. Two endometriosis samples showed no variants (Tables S1 and S2).

The most frequently mutated genes in ovarian endometrioid carcinoma patients were *TP53, KDR, MET*, *PIK3CA, FGFR3* and *PTEN*, while in endometriosis the most frequently mutated genes were *KDR, AKT1, MET* and *PIK3CA*. **Figure 1** along with the Tables S1 and S2 present the number of mutated samples per gene and the number of variants identified in each gene.

Mutation validation

Two variants that were exhibited in more than one patient with ovarian endometrioid carcinoma were tested for validation; the exact gene

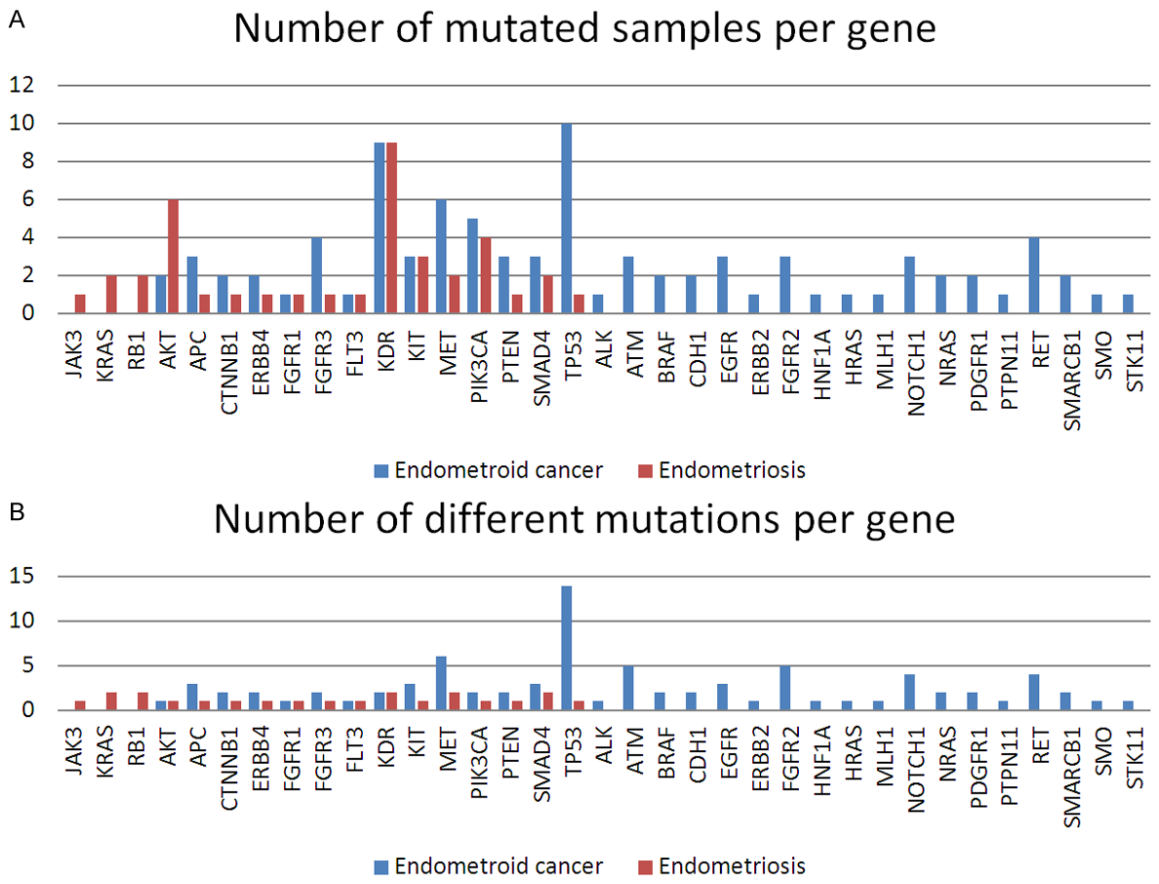

Figure 1. A. Bar graph comparing the two patient cohorts in terms of the number of mutated samples per each gene; B. Bar graph comparing the two patient cohorts in terms of the number of different variants identified for each gene.

coding variants tested using the TaqMan SNP genotyping assay (AKT1 rs3730358, assay ID C____193157_10, KDR c.1416T>A, rs1870377, assay ID C__11895315_20). It was determined that the variants *AKT1* rs370358 and *KDR* c.1416A>T were properly validated. For endometriosis samples the following TaqMan SNP assay were chosen for validation: AKT1 rs3730358, assay ID C__193157_10, KDR c.1416T>A, rs1870377, assay ID C__11895315_20 and KIT rs1870377 custom assay. All assays were properly validated and the percentage of positive samples was similar with the percentage obtained in the test samples.

BRCA mutation identification

58 different variants were identified from the endometriosis patient, 41 of which were not yet identified in any other sample databases and classified as Class 3 by ClinVar clasifications. No class 5 variants were identified in this group of patients (as seen in **Figure 2B** and Table S3).

The ovarian endometrioid carcinoma cohort exhibited 100 different variants, five of which were Class 5 variants, all in BRCA1. In addition, in this cohort of patients, the majority of variants (74) were Class 3, meaning variants that are either not identified yet or classified as such in ClinVar (**Figure 2A** and Table S4).

Despite most of the patients in both groups presenting both *BRCA1* and *BRCA2* variants, the ovarian endometrioid carcinoma group did contain 3 patients with no *BRCA1/2* variants. **Figure 2C** and **2D** presents the percentage of variants in either *BRCA1* or *BRCA2* exhibited in either patient cohort.

There were two Class 3 variants common for both groups of patients: one ovarian endometrioid carcinoma sample and one endometriosis sample had *BRCA1* c.2501G>A identified;

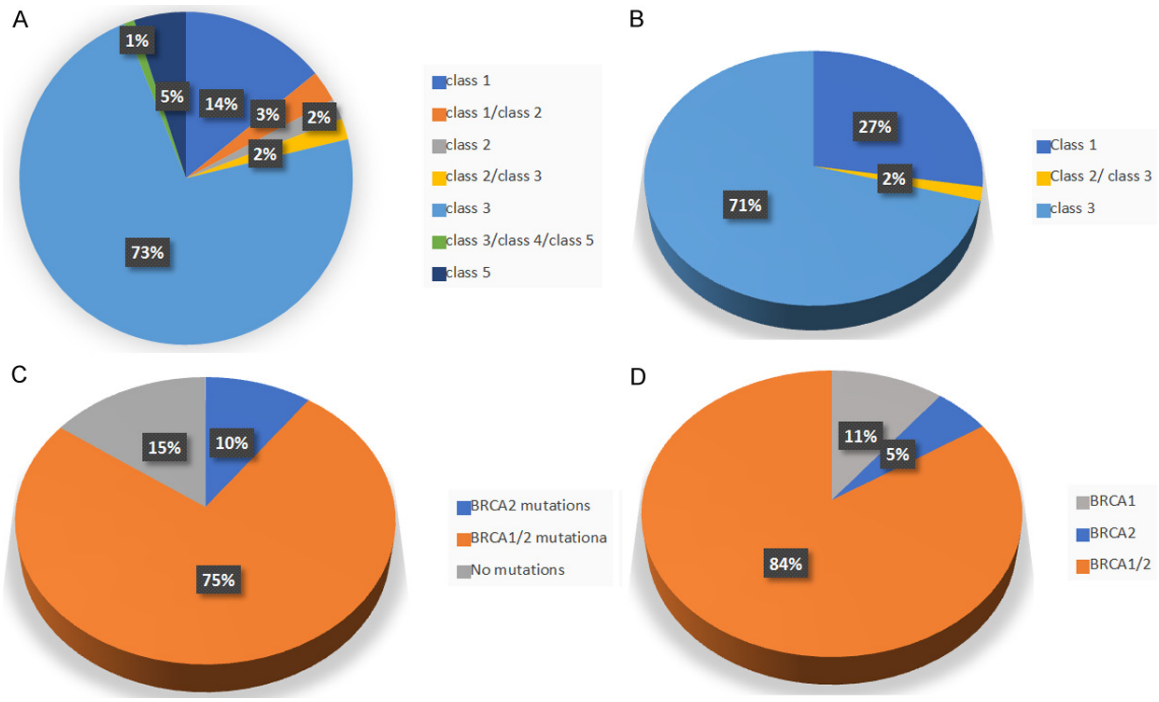

Figure 2. Pie graphs depicting the percentage of each pathogenicity class defined by ClinVar for the identified mutations. (A) Samples from the endometrioid cancer patients; (B) Samples from the endometriosis patients. Pie graphs of the percentage of variants classified by the type of mutated gene for: (C) ovarian endometrioid cancer patients; (D) endometriosis patients.

and *BRCA1* c.441+50del14 was identified in one ovarian endometrioid carcinoma sample and in six endometriosis samples.

Identification of the methylation profile

The methylation profile and copy number aberration experiments were done on 21 endometriosis samples and 20 ovarian endometrioid carcinoma samples. We evaluated 45 genes and observed different methylation profiles in 36 genes for endometrioid ovarian carcinoma and 38 for endometriosis However, we were only able to obtain methylation profiles for 19 of the ovarian endometrioid carcinoma samples; the remaining one ovarian endometrioid carcinoma sample presented DNA with quality and quantity not suited for this experiment. No viable results could be obtained. The aforementioned results obtained for the remaining samples are presented in Tables S5 and S6. In **Figure 3A** are presented the number of endometrioid ovarian carcinoma samples and endometriosis samples that presented methylation in the tested genes. As can be seen there are some genes that are highly methylated only in endometriosis samples (*PYCARD, RARB, RB1, IL2, CFTR, CD44* and CDH13) and MLH3 gene was methylated only in endometrioid ovarian carcinoma samples. Also, *BRCA1, CADM1, PAX6* and *PAH* genes are mainly methylated in endometrioid ovarian carcinoma patients. The highly methylated genes in endometrioid ovarian carcinoma are *BRCA1, BRCA2, TP53, TP73, PAX5, GSTP1, MSH6, STK11, KLLN, PTCH1, TSC2* and *VHL*. Also the *CFTR* gene was hypomethylated in almost all tested sample, except one (**Figure 3A**).

The methylation status was correlated to the differentiation of the tumor grade (G1, G2, G3) of ovarian endometrioid carcinoma patients, which can be seen in **Figure 3B**. It was observed that: all patients with G2 tumor grade exhibited methylation in *WT1,* CHFR, *MSH6, PTCH1* and *TSC2* genes and did not present methylation in *CD44, CDKN2, CFTR* and *RARB.* Patients with G3 tumors showed no methylation for *KLK2, RARB* and *RB1* genes, and patients with G1 tumors did not show methylation in *CDKN2A, MLH3* and *CFTR.* It should be noted that no tumors extracted from the patients exhibited the tumor grade Gx or G4. Regardless of the differentiation degree of the

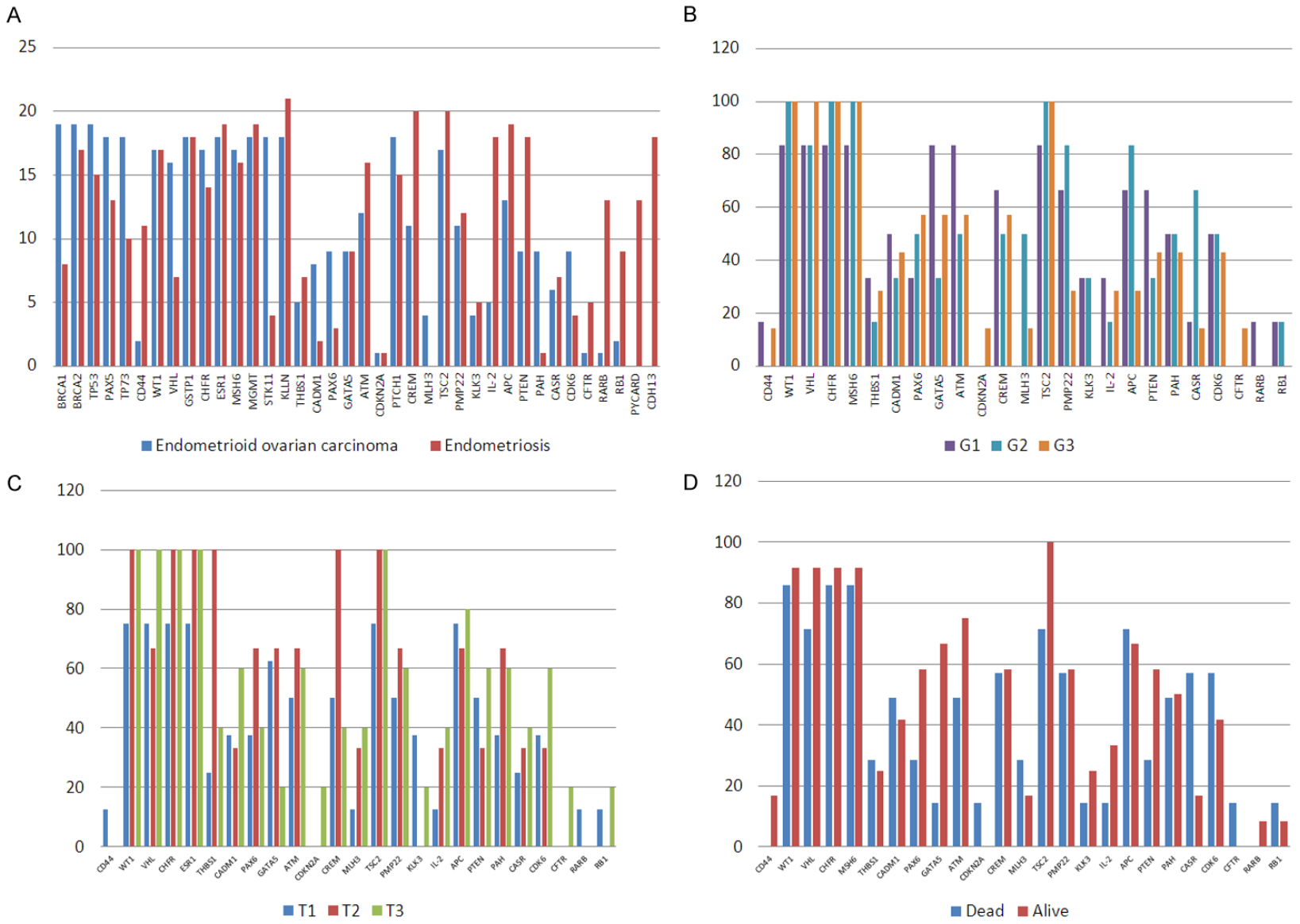

Figure 3. Bar graph comparing the (A) No of methylated endometrioid ovarian cancer (19) and endometriosis samples (21). Bar graph comparing the percent frequency of methylation of the 20 selected genes with (B) tumor differentiation grade (C) tumor stage; (D) Bar graph comparing the percent frequency of methylation of the 20 selected genes pre- and post-mortem, exhibited in the ovarian endometrioid carcinoma patients (n=19).

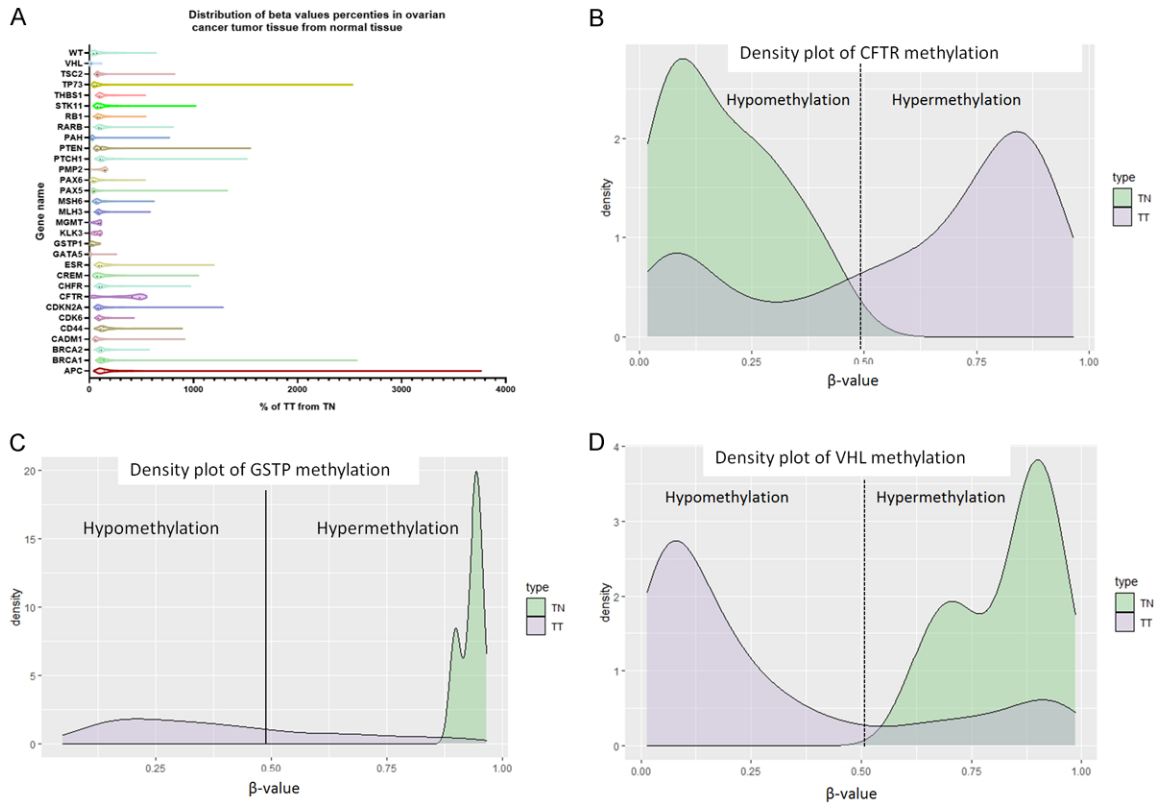

Figure 4. Methylation data for ovarian cancer patients from TCGA data. (A) distribution of beta values, (B) density plot for *CFTR* gene, (C) Density plot for *GSTP* gene, (D) density plot for *VHL* gene.

tumor we observed that all samples were methylated in the following genes: *BRCA1, BRCA2, TP53, PAX5, TP73, GSTP1, ESR1, MGMT, STK11, KLLN* and *PTCH1*.

Subsequently, the methylation was correlated to the cancer stage of the main tumors extracted from patient groups (T1-T3), which can be seen in **Figure 3B**. Firstly, all patients with T2 tumors had *WT1, CHFR, ESR1, THBS1, CREM and TSC2* methylated. In addition, the T2 tumors from patients did not present hypermethylation in *CD44, CDKN2A, KLK3, CFTR, RARB and RB1*. Secondly all T3 tumors had *WT1, VHL, CHFR, ESR1 and TSC2* methylation-and did not show methylation for *CD44* and *RARB* genes. Thirdly, *BRCA1, BRCA2, TP53, PAX5, TP73, GSTP1, MSH6, MGMT, STK11, KLLN* and *PTCH1* were methylated in all samples regardless of the cancer stage. No tumors extracted from the patients exhibited a cancer stage of T4.

Lastly, *CDKN2A* and *CFTR* hypermethylation was observed in the same deceased patient at the time of the experiments, whereas *CD44* hypermethylation was observed in two alive patients (**Figure 3D**). Also, all alive patients showed *TSC2* hypermethylation, whereas only 71% of deceased patients presented this hypermethylation. *BRCA1, BRCA2, TP53, PAX5, TP73, GSTP1, ESR1, MGMT, STK11, KLLN* and *PTCH1* hypermethylation was observed in all patients regardless if they were alive or dead.

TCGA data for methylation and mutation validation in ovarian cancer

The TCGA data from UCSC Xena database was used for the validation of the methylation profile of ovarian cancer patients, this data set contains methilation profile on 168 genes, from which we selected only the genes that we evaluated in our cohort of patients. As it can be observed in **Figure 4A** *APC, TP73, BRCA1, PTEN, PTCH1, PAX5, CDKN2A* and *ESR* showed the higher methylation profile. As can be seen in **Figure 4B**, *CFTR* gene is hypermethylated in the TCGA data, whereas *GSTP* (**Figure 4C**) and *VHL* (**Figure 4D**) genes are hypomethylate as

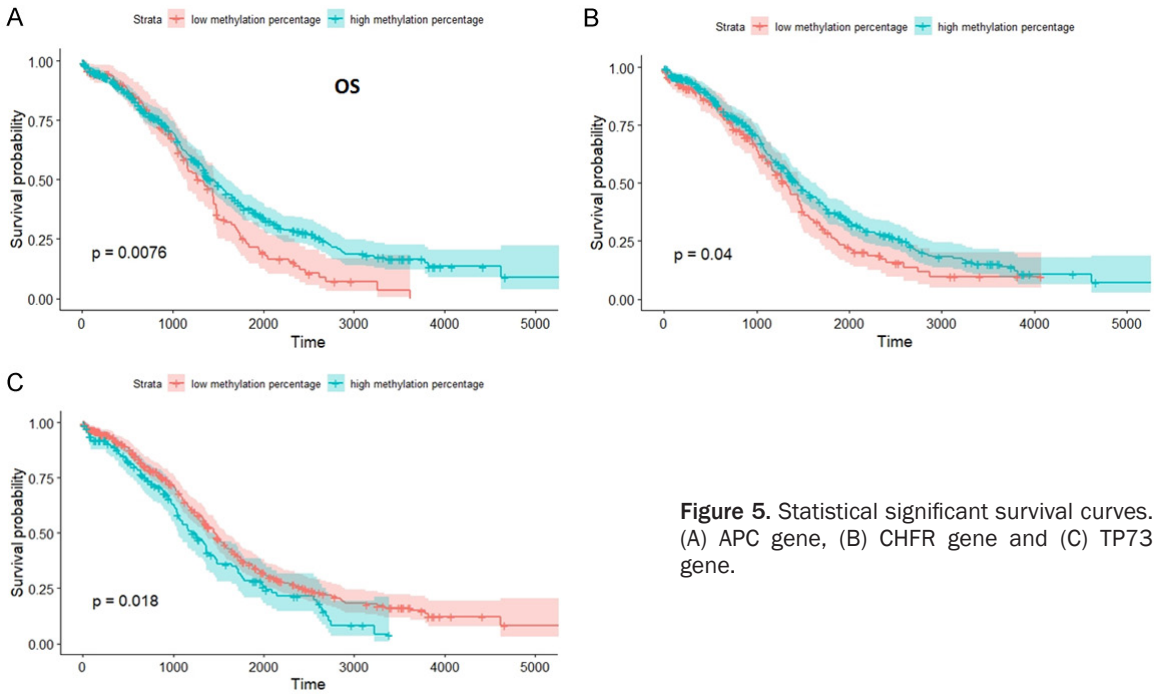

Figure 5. Statistical significant survival curves. (A) APC gene, (B) CHFR gene and (C) TP73 gene.

compared to normal samples. This result could be attributed to the fact that in TCGA data we have all ovarian cancer, not only endometrioid ovarian cancer.

We analyzed the survival data for the studies samples in TCGA database and obtained statistical significant data for *APC, CHFR* and *TP73* genes. As can be seen in **Figure 5** patients with hypermethylation in APC and CHFR gene have a better survival rate that those with hypomethylation, while in the case of TP73 patients with hypomethylation have better survival rates that those with hypermethylation.

Regarding the mutation profile of ovarian cancer samples from TCGA data we observed that the most frequently mutated gene in this cohort is *TP53*, followed by *RB1, KDR, RET, ATM, APC, ALK* and *PIK3CA*. As can be observed in **Figure 6** the TCGA ovarian cohort has comparable mutation percentages in some genes both with endometrioid ovarian cancer and endometriosis.

Statistical analysis

Tables 2 and **3** show the statistical correlation between the validated gene variants (pathogenic *BRCA1*, *TP53* variants or *AKT* rs3730358) and the clinical information pertaining to the ovarian endometrioid carcinoma patients (tumor differentiation grade, tumor stage, metastases or copy number aberrations). The values in bold represent correlations which are statistically significant.

A statistically significant inverse correlation was observed between: the presence of *BRCA1* pathogenic variants and the differentiation grade of the tumors; the presence of *BRCA1* pathogenic variants and copy number aberrations; and lastly, copy number alterations and metastases. There was a statistically significant direct correlation between: the presence of *BRCA1* pathogenic variants and *TP53* variants; tumor stage and copy number aberrations.

In the case of *p*-value correlations, the tumor differentiation grade directly correlated with the tumor stage and the presence of metastases. Tumor stage was also correlated with the presence of metastases and mortality. Lastly, copy number aberrations were statistically correlated with the presence of *TP53* variants.

We compared the survival curves for cancer patients that showed the validated variants (*AKT, KDR* or pathogenic *BRCA1* variants) and the group of patients that did not show these variants, but no statistically significant correlation was found. In addition, when the survival curves for patients that presented copy num-

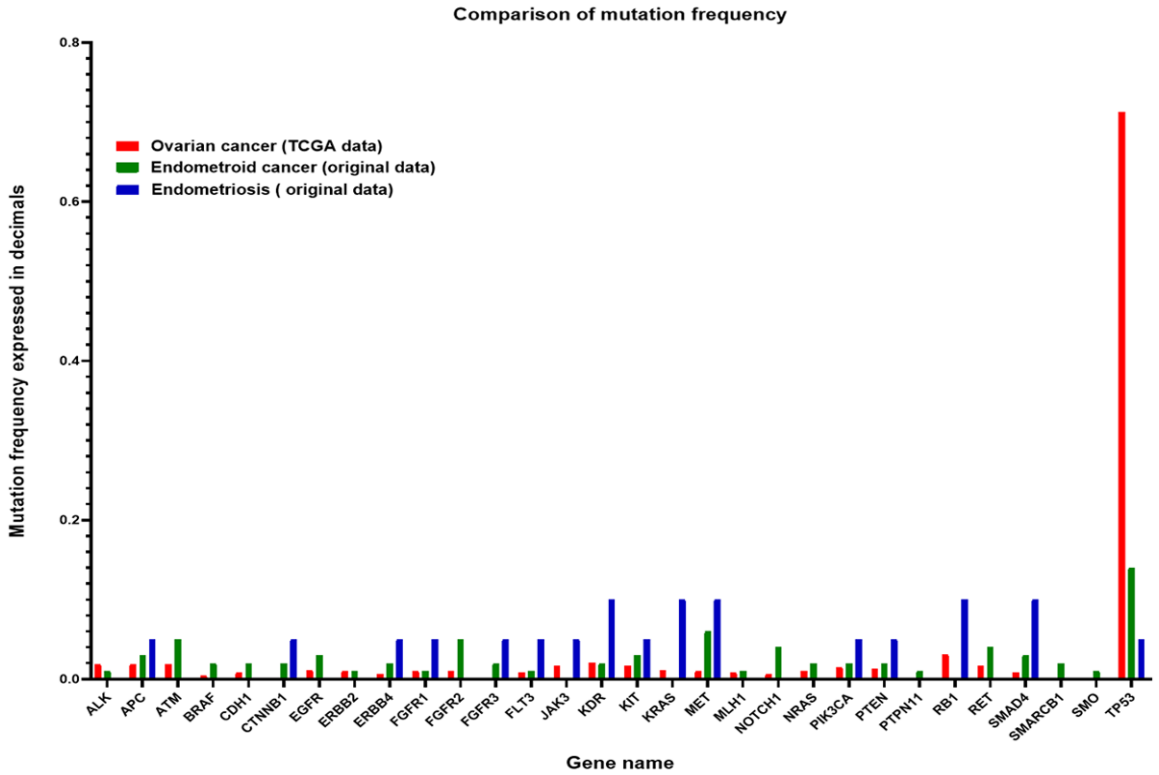

Figure 6. Boxplot of mutations frequencies for ovarian cancer (TCGA data), endometrioid ovarian cancer (original data) and endometriosis (original data).

Table 2. Correlation coefficients (r) for ovarian endometrioid carcinoma patients between the following criteria: pathogenic variant of BRCA1, tumor differentiation grade, tumor cancer stage, TP53 variant and copy number aberrations

	BRCA1 pathogenic	Tumor Differentiation Grade	Tumor stage	Metastases	TP53 variants	Copy number aberrations
BRCA1 pathogenic		**-0.03**	**-0.17**	**-0.22**	**0.00**	**-0.05**
Tumor Differentiation Grade	**-0.03**		0.53	0.56	**-0.37**	0.27
Tumor stage	**-0.17**	0.53		0.71	**-0.13**	**0.001**
Metastases	**-0.22**	0.56	0.71		**-0.22**	**-0.05**
TP53 variants	**0.001**	**-0.37**	**-0.13**	**-0.22**		0.44
Copy number aberrations	**-0.05**	0.27	**0.001**	**-0.05**	0.44	

Table 3. Correlation of criteria in terms of *p*-value for ovarian endometrioid carcinoma patients

	AKT rs3730358	Tumor Differentiation Grade	Tumor stage	Metastases	Mortality	TP53 variants	Copy number aberrations
AKT rs3730358		0.44	0.44	0.36	0.07	0.15	0.54
Differentiation Grade	0.44		**0.04**	**0.02**	0.13	0.14	0.29
Tumor stage	0.44	**0.04**		**0.001**	**0.03**	0.62	1.00
Metastases	0.36	**0.02**	**0.001**		0.18	0.36	0.84
Deceased	0.07	0.13	**0.03**	0.18		0.07	0.71
TP53 variants	0.15	0.14	0.62	0.36	0.07		**0.05**
Copy number aberrations	0.54	0.29	1.00	0.84	0.71	**0.05**	

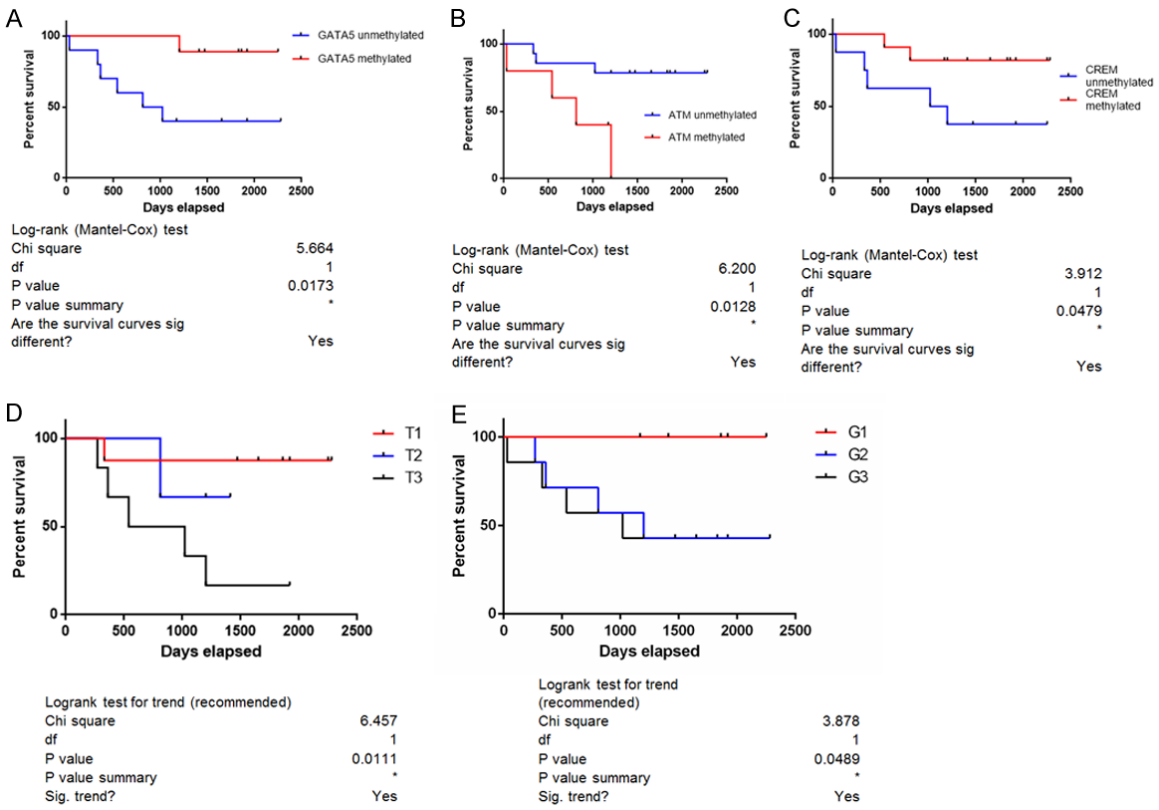

Figure 7. Kaplan-Meier Survival Curves for the ovarian endometrioid carcinoma patients in correlation to (A) *GATA5* hypermethylation; (B) ATM hypermethylation; (C) CREM hypermethylation; (D) tumor stage; (E) tumor differentiation grade.

ber aberrations were analyzed no statistically significant results were obtained. The same was true in the case of the patients with *AKT* rs3730358 and *KDR* rs1870377 variants. **Figure 7** presents the survival curve with a statistically significant value, which was in the case of the hypermethylation of *GATA2, CREM* and *ATM* gene, tumor stage and tumor differentiation grade.

Gene ontology

For Gene Ontology, we used the Panther DB online software in order to evaluate the processes involving the mutated and methylated genes. **Figure 8** presents the gene ontology analysis for the endometriosis and endometrioid ovarian cancer patients, also in terms of the different methylation profiles exhibited.

Discussion

Principal findings

Our study showed that there are significant differences between endometriosis and the ovarian endometrioid carcinoma samples regarding the mutation profile for *BRCA1/2* and the 46 genes involved in cancer. As can be seen in **Figure 1**, there are specific gene variants expressed for the endometriosis patients (*JAK3*, *KRAS*, *RB1*), some variants can only be found in the ovarian endometrioid carcinoma patients (*ALK, ATM, BRAF, CDH1, EGFR, ERBB2, FGFR2, HNF1A, HRAS, MLH1, NOTCH1, NRAS, PDGFR1, PTPN11, RET, SMARCB1, SMO, STK11*), while others *PIK3CA*, *AKT, KDR* and *PTEN* are common for the two groups. Regarding the methylation profile, we observed that *ATM* hypermethylation was correlated with a poor survival, whereas patients with hypomethylated GATA5 and CREM genes show lower survival rates, and that there are specific hypermethylation profiles correlated with tumor size and the differentiation degree of the tumor.

One weakness of our study is related to the low number of patients tested, and to the fact that the quantity and quality of DNA from endometriosis patients was low.

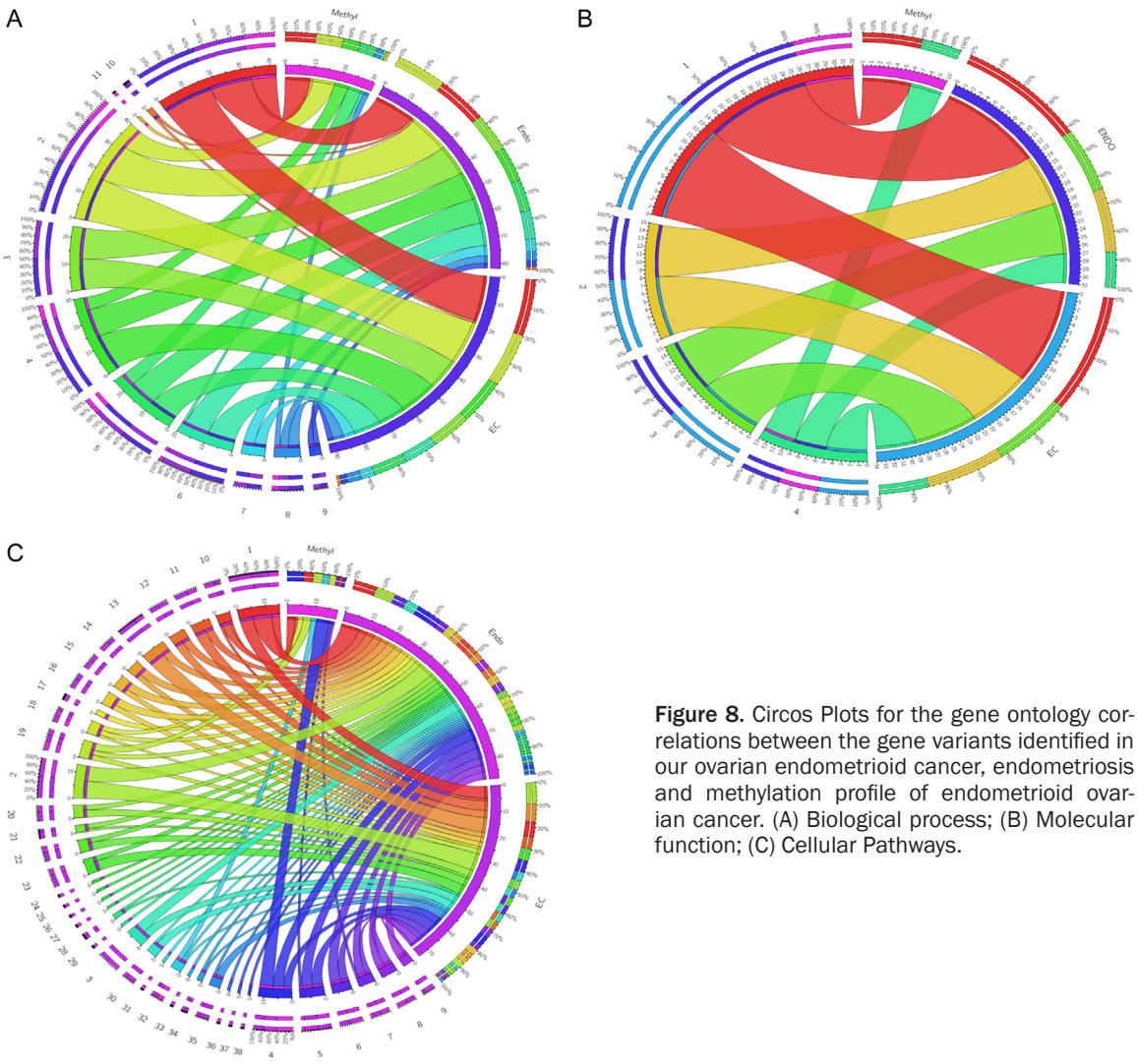

Figure 8. Circos Plots for the gene ontology correlations between the gene variants identified in our ovarian endometrioid cancer, endometriosis and methylation profile of endometrioid ovarian cancer. (A) Biological process; (B) Molecular function; (C) Cellular Pathways.

We observed that endometriosis patients did not show any *BRCA1/2* pathogenic variants, but showed some *BRCA1/2* variants that are not yet known to have any clinical significance. In contrast, in the case of patients with ovarian endometrioid carcinoma, there were four samples (20%) that presented pathogenic *BRCA1* variants, of which one sample had two variants (c.3544C>T, c.3607C>T, c.1115G>A, c.4612C>T and c.2563C>T).

In the case of the mutation profile of the 46 genes tested, the endometriosis samples presented fewer variants than the ovarian endometrioid carcinoma samples. More specifically, these samples had nine variants identified and classified as pathogenic by the FATHMM score in COSMIC database. Of these, only two variants were present in more than one sample, namely *KIT* c.1621A>C (3 samples) and *PIK-3CA* c.2119G>A (4 samples). The remaining majority of the other variants were new. It should be noted that two endometriosis samples did not show any variants. On the other hand, the ovarian endometrioid carcinoma samples had variants identified in 31 genes, and 43 of the identified mutations were already known; most were classified as pathogenic by the FATHMM score in the COSMIC database.

The percentage of patients with *BRCA1/2* variants found in our study is similar to the reported percentage in the literature [19]. All the pathogenic variants identified in our study were identified in the ClinVar database, where they were associated with familial breast and ovarian cancer predisposition syndrome. The

c.4612C>T mutation is associated with ovarian cancer as described by Yang et al. [20].

Regarding the ovarian endometrioid carcinoma samples, pathogenic variants were observed in several genes, from which two genes presented the same pathogenic variants in more than one samples: *CTNNB1* c.110C>G (2 samples) and *PIK3CA* c.2119G>A (3 samples).

Moreover, the specific mutations *PIK3CA* c.2119G>A and *KIT* c.1621A>C were observed in both of our patient cohorts. Both of these mutations are exhibited in triple negative breast cancer patients [18], which is an interesting correlation to further investigate: the predisposition of ovarian endometrioid carcinoma to present similar mutations as triple negative breast cancer.

In accordance with the literature [19], 50% of the ovarian endometrioid carcinoma samples exhibited *TP53* variants. By analyzing TCGA data sets we observed a correlation between the percentage of *TP53* mutations in our samples and the TCGA database. Additionally, it is well known from the literature that the ovarian endometrioid carcinoma is associated with variants in the *PTEN* gene and in the PI3K pathway [21]. Our data also supports this association. More specifically, three of our samples presented variants in *PTEN* gene and five samples in *PIK3CA* gene, which could make these patients susceptible to PARP-PI3K inhibitor as described by Bian et al. [22]. Also, when we used the inTogen database to evaluate which genes are considered as drivers in ovarian cancer, the main gene is *TP53*, followed by *BRCA1, BRCA2* and *RB1*, which are genes commonly mutated in our cohort of patients, as well as in the TCGA database [23].

Aberrant methylation of CpG islands is correlated with silencing or different expression of tumor suppressor genes in cancer. In our cohort of cancer patients, we observed abnormal methylation in several genes including *BRCA1/2, TP53, TP73, PAX5, ESR*, *MSH6, PTCH1, PTEN* and others (refer to Table S5), which were also hypermethylated in the TCGA cohort. As described in the literature, *BRCA1* gene is hypermethylated only in breast and ovarian cancer [24]. Ibanez de Caceres et al. observed that 82% of patients with ovarian cancer presented hypermethylation in one of the following genes: *BRCA1*, *RASSF1A, p14-ARF, APC, p16INK4A* and *DARK* [25, 26]. We observed hypermethylation in 19 of the 20 patients that were tested, which may be due to the fact that the promoter regions of 25 genes tested are frequently methylated in cancer. Melnikov et al. showed that ovarian cancer patients show increased methylation in genes like *BRCA1, EP300, NR3C1, MLH1, DNAjC15, CDKN1C, TP73, PGR, THBS1, PAX5* and *PYCARD* [27]. Interestingly, we observed methylation deregulations in some of the genes presented in Melnokov's paper, such as *BRCA1, TP73, THBS1* and *PAX5*. It was observed that hypermethylation in ovarian cancer inactivates pathways like DNA repair, cell cycle regulation, cell adhesion and apoptosis, all of which are involved in ovarian cancer development [28-30].

The patients with G2 or G3 tumors showed lower survival rates compared to patients with G1 tumors as seen in **Figure 7E**, which is in accordance with the literature [31, 32]. Also, it was observed that patient survival is similar in the concurrent ovarian endometrioid carcinoma and endometrial endometrioid carcinoma stage I [33].

When doing Gene ontology analysis for the different mutated genes in ovarian endometrioid carcinoma and endometriosis patients, it was observed that in both diseases the same biological processes and molecular functions were present. Concerning the pathways in which the mutated genes were involved, the main ones were angiogenesis, apoptosis, p53 pathway, PI3 kinase, EGF signaling, VEGF and TGF signaling. The same pathways were observed in both types of patients; exemplified in the Circos Diagrams of **Figure 8**. Analysis of the Gene ontology results for the genes with different methylation profiles determined that only two molecular functions were re-established: firstly, binding and catalytic activity; and secondly, the pathways were correlated to the p53 protein. Also, the presence of deregulated inflammatory and RAS protein pathways was observed in both groups studied, which correlates with the recent literature [34]. Regarding the other pathways, the presence of apoptosis and WNT signaling were also identified (refer to **Figure 8** and **Table 4**). Taking all of this into consideration, we can say that based on common pro-

Table 4. Table legend for **Figure 8**

Code	**Figure 8A**	Code	**Figure 8B**
1	cellular process (GO:0009987)	1	binding (GO:0005488)
2	metabolic process (GO:0008152)	2	receptor activity (GO:0004872)
3	biological regulation (GO:0065007)	3	signal transducer activity (GO:0004871)
4	response to stimulus (GO:0050896)	4	catalytic activity (GO:0003824)
5	developmental process (GO:0032502)		
6	multicellular organismal process (GO:0032501)		
7	localization (GO:0051179)		
8	cellular component organization or biogenesis (GO:0071840)		
9	locomotion (GO:0040011)		
10	biological adhesion (GO:0022610)		
11	reproduction (GO:0000003)		
code	**Figure 8C**	code	**Figure 8C**
1	p53 pathway feedback loops 2 (P04398)	20	T cell activation (P00053)
2	Angiogenesis (P00005)	21	Endothelin signaling pathway (P00019)
3	Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	22	PDGF signaling pathway (P00047)
4	p53 pathway (P00059)	23	Cadherin signaling pathway (P00012)
5	Wnt signaling pathway (P00057)	24	DPP signaling pathway (P06213)
6	FGF signaling pathway (P00021)	25	DPP-SCW signaling pathway (P06212)
7	Apoptosis signaling pathway (P00006)	26	BMP/activin signaling pathway-drosophila (P06211)
8	Interleukin signaling pathway (P00036)	27	Activin beta signaling pathway (P06210)
9	Insulin/IGF pathway-protein kinase B signaling cascade (P00033)	28	Axon guidance mediated by netrin (P00009)
10	Hypoxia response via HIF activation (P00030)	29	JAK/STAT signaling pathway (P00038)
11	VEGF signaling pathway (P00056)	30	Integrin signalling pathway (P00034)
12	Ras Pathway (P04393)	31	ALP23B signaling pathway (P06209)
13	EGF receptor signaling pathway (P00018)	32	P53 pathway feedback loops 1 (P04392)
14	PI3 kinase pathway (P00048)	33	FAS signaling pathway (P00020)
15	CCKR signaling map (P06959)	34	TGF-beta signaling pathway (P00052)
16	Gonadotropin-releasing hormone receptor pathway (P06664)	35	B cell activation (P00010)
17	Alzheimer disease-presenilin pathway (P00004)	36	SCW signaling pathway (P06216)
18	Huntington disease (P00029)	37	MYO signaling pathway (P06215)
19	p53 pathway by glucose deprivation (P04397)	38	GBB signaling pathway (P06214)

cesses, molecular functions and pathways that overlap in both endometriosis and ovarian endometrioid carcinoma, a diagnosis of endometriosis could be utilized as an early sign of ovarian endometrioid carcinoma for the respective patients. This notion has been argued and supported in the literature, stating that ovarian endometrioid carcinoma particularly originates from ovarian or pelvic endometriosis [35]. Due to the fact that gene ontology analysis of methylation data showed such specific pathways, one can conclude that ovarian endometrioid carcinoma is strongly correlated with p53 and apoptosis deregulations. Furthermore, other studies extend this idea to any cellular processes related to regulation, development and interaction [36, 37].

Conclusion

In conclusion, this study was able to demonstrate the presence of the common signaling pathways and molecular functions, supported by similar genetic network alterations, in both endometriosis and ovarian endometrioid carcinoma. This further drives the notion that the diagnosis of endometriosis could be an early marker for ovarian endometrioid cancer diagnosis. Lastly, it was demonstrated that *GATA2* hypomethylation, *ATM* hypermethylation, *CREM* hypomethylation, higher tumor differentiation degree or higher tumor stage is associated with a poor prognosis in patients with ovarian endometrioid carcinoma.

Disclosure of conflict of interest

None.

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