

EXPRESSION OF PROAPOPTOTIC-ANTIPOPTOTIC GENES IN MALIGNANT, BORDERLINE AND BENIGN OVARIAN TUMORS

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ABSTRACT: There is a wide group of ovarian tumors, malignant as well as benign. Histopathological examination is used as a primary source of identifying the difference between malignant and benign processes. We analyzed ovarian tissue samples of twelve women, ages ranging from 21 to 77. Samples of 8 benign, 1 borderline, and 3 malignant ovarian tumors were included in the study. Using the quantitative PCR method, genes indicating apoptotic processes in tissue cells were evaluated, based on monitoring their relative expression. Relative gene expression was monitored for anti-apoptotic protein *Bcl-2*, pro-apoptotic protein *Bax*, and effector protein *Cas3*. Expression of gene for protein *Bax* and *Bcl-2* is increased and *Bax* predominates, which indicates that the initiation of apoptosis has begun, mostly in benign tumors. *Bcl-2* was elevated in samples of borderline and malignant tumors while *Bax* was decreased, which indicates the inhibition of apoptosis in these samples.

Keywords: *bcl-2*, *bax*, ovarian tumor, ovarian carcinoma, apoptosis.

INTRODUCTION

Ovarian tumors represent a specific problem in gynecology, because of their lack of clear symptomatology. It is known that when it comes to ovarian tumors, the difference between benign and malignant processes is hard to determine.

Benign ovarian tumors are mostly asymptomatic, appear in younger women, and are mostly functional ovarian cysts, dependent on the hormone fluctuations of the menstrual cycle and thus regress spontaneously over time. These cysts are formed during a reproductive time in women's lives and are associated with the process of ovulation. They do not represent neoplasms in a real sense, because they are created from follicles or corpus luteum and grow due to secretion in an already existing natural cavity (PLEČAŠ *et al.*, 2011).

The most common benign epithelial tumors are ovarian cystadenomas, serous subtype. (LIMAIEM *et al.*, 2023) They are most expected between 40 to 60 years of age but can be found in any age group (WEBB and JORDAN, 2017). A sub form of a serous cystadenoma is cystadenofibroma, containing cystic and solid parts. Mucinous cystadenoma account for a quarter of all benign ovarian tumors, mainly during 30 to 60 years of age, but are not uncommon in younger women. They grow to extreme dimensions and can weigh up to 20 kg (PLEČAŠ *et al.*, 2011). Histopathological examination is still used as a primary source of identifying cystadenomas, despite advancements in imaging methods. Almost all malignant tumors are classified into three main categories. This classification is based on histogenesis, by which primary ovarian tumors are divided into epithelial-type tumors, germ cell tumors, and sex chord stromal type tumors (HOFFMAN *et al.*, 2021).

Epithelial ovarian cancers (EOCs) represent a vast majority of all ovarian cancers. By histological subtypes they are divided into two groups. Tumors from the first group grow slowly, often from an identifiable precursor. In contrast, tumors from the second group are high-grade and rapidly progressive. Lack of specific symptoms, unclear etiology and heterogeneity of OC contribute to delayed diagnosis and overall poor survival rates (LHEUREUX *et al.*, 2019).

Currently, there are a few biomarkers used for OC management, CA-125 being the most promising one (EINHORN *et al.*, 1992; ZWAKMAN *et al.*, 2017). However, menstruation, endometriosis and other inflammatory diseases are associated with increased CA-125 levels, thus it lacks in specificity.

New studies are attempting full gene expression profiling as a way to discover potential molecular biomarkers capable of improving tumor classification and staging, predicting chemotherapy response, and impacting overall patient outcome (KONSTANTINOPOULOS *et al.*, 2008; LEONG *et al.*, 2015; WINTERHOFF *et al.*, 2016).

The biggest vulnerability cells have to apoptosis is determined by ratios of apoptotic-antiapoptotic genes (OLTVAI *et al.*, 1993). It has been shown that levels of expression of Bcl-2 and Bax can be a prognostic factor in OC (SCHUYER *et al.*, 2001; DE LA TORRE *et al.*, 2007). The main goal in this study was to detect different levels of expression of apoptotic-antiapoptotic genes in different types of ovarian tumors, malignant as well as benign.

MATERIALS AND METHODS

Patients

In this study, tissue samples were obtained from patients who underwent surgery for different gynecological symptomatology from 2019-2020 in the University Clinical Center of Kragujevac, Clinic of Gynecology and Obstetrics. Twenty-eight ovarian tissue specimens were subtracted from twelve women. The samples consisted of 8 benign (2 cystadenofibromas (O3T, O8T), 2 endometriotic cysts (O6T, O7T), 1 cystadenoma serous (O5T), 1 cystadenoma mucinous (O9T), 1 teratoma (O12T) and 1 inclusion cyst (O10T), 1 borderline (mucinous (O1T), and 3 malignant ovarian tumors (all 3 serous (O2T,O4T,O11T). The accordance of histopathological diagnosis and findings collected during clinical follow-up was evaluated by reviewing patients' charts before categorizing cases to tumor types.

Total RNA isolation

Tissue homogenization

Tissue samples were homogenized with 5 mL of PBS in a metal ball homogenizer. Homogenization was performed at maximum speed three times for 30 seconds. The entire

contents were transferred to a 15 mL test tube and centrifuged at 500 rpm/5 min at room temperature to remove tissue debris (ŽIVANOVIĆ *et al.*, 2023).

Isolation

Total RNA isolation was performed by a standard method using TRIzol® reagent. TRIzol® reagent and chloroform were added to each sample. Mixture is well vortexed and left at room temperature for 2-3 minutes. After that, the mixture is centrifuged at 15000 RCF/15 min at 4°C. Three phases were separated by centrifugation: RNA is found in the upper clear phase, proteins are found in the lower red phase, while DNA is separated in the interphase like a white track. The clear phase containing the RNA is carefully separated and transferred to another microtube, isopropanol is added to precipitate the RNA, vortexed well and left at room temperature for 10 minutes. The mixture is centrifuged at 12000 RCF/10 min at 4°C. The supernatant is discarded, and the RNA, which remained in the precipitate, is washed with 70% ethanol and centrifuged for 5 min/7000 RCF/4°C twice. After drying and evaporating the ethanol, on a thermoblock, total RNA was dissolved in 30 µL of water for injection and 5 µL of RNA later was added. The concentration of isolated RNA was measured using a MultiskanSkyHigh UV/VIS spectrophotometer on the wavelength of 260 nm and purity was measured on as a ratio of wavelength 260 nm/230 nm and 260nm/280 nm (ŽIVANOVIĆ *et al.*, 2023).

Translation of RNA into complementary DNA, reverse transcription

Translation of total RNA into complementary DNA (cDNA) was performed using the NG dART RT Kit on an Multiskan Optimax, Labnet International according to the manufacturer's instructions. The total volume of the reaction mixture was 20 µL, and it contained 4 µL 5x NG.

cDNA Buffer, 1 µL Oligo(dT)20 primer, 1 µL NG dART RT Mix, total template RNA at an amount of 0.5 µg and RNase-free water to the total volume. The reaction was carried out in several steps. Primer binding and reverse transcription was performed at a temperature of 50°C for 45 minutes. The terminal phase of the reaction is incubation at 85°C for 5 minutes, followed by cooling to 4°C. In this way cDNA was obtained, which is more stable than RNA and can be stored at - 80°C (ŽIVANOVIĆ *et al.*, 2023).

Quantitative polymerase chain reaction (qPCR)

Quantitative PCR was used to determine the relative expression of apoptosis-related genes. The reaction was performed using the SG/ROX onTaq qPCR Master Mix kit (2x) on the apparatus Gentier 96, Tianlong® according to the manufacturer's protocol. The total volume of the reaction mixture was 25 µL. The reaction mixture was made using 12.5 µL SG/ROX onTaq qPCR Master Mix (2x), Forward and Reverse primers at the concentration of 0.5µM, cDNA at an amount of 450 ng and nuclease free water to the total volume.

The reaction was carried out in several steps. The first step is initial incubation at 95°C for 15 minutes. This is followed by 3-step amplification, which includes denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, in 40 cycles. Next step was melting. Melting includes 3 steps, and their parameters are 95°C/1 min, 60°C/15 s, 98°C/5 s, after which cooling to 4°C occurs. Genes of interest and corresponding primers are shown in Tables 1 and 2.

The housekeeping gene represents some of the constitutive genes necessary to maintain the basic functions of the cell. These genes are expressed in physiological and pathophysiological conditions. Here, β-actin was used as a housekeeping gene because it showed least error in variation among housekeeping genes in samples like these (NOROUZI-

BAROUGH *et al.*, 2018). Relative expression levels are calculated according the $2^{-\Delta\Delta Ct}$ method (LIVAK and SCHMITTGEN, 2001).

Table 1. Primers for qPCR.

Gene	Primer sequence
<i>Bcl - 2</i>	F 5'- gataacggaggctgggatgc -3'
	R 5'- gacttcacttgtggcccagat -3'
<i>Bax</i>	F 5'- gcttcagggttcatccagga -3'
	R 5'- caatcatcctctgcagctcca -3'
<i>Cas 3</i>	F 5'- gcacctggttattattcttggc -3'
	R 5'- ggactcaaattctgttccacc -3'
β -actin	F 5'- ctcacctgaagtacccatc -3'
	R 5'- aggtctcaaacatgatctggg -3'

Table 2. qPCR target information.

Target name	Abbreviation	Accession number/Locus	Amplicon length	Secondary structures
B-cell CLL/ lymphoma 2	<i>Bcl-2</i>	NM_000633.3	154 bp	No
Bcl-2-associated X protein	<i>Bax</i>	NM_138761.4	164 bp	No
Caspase 3	<i>Cas3</i>	NM_004346.4	150 bp	No
Beta-actin (Housekeeping)	β -actin	NM_001101.5	1812 bp	Very weak

RESULTS

Using the quantitative PCR method, genes indicating apoptotic processes in tissue cells were evaluated, based on monitoring their relative expression. Relative gene expression was monitored for anti-apoptotic protein *Bcl-2*, pro-apoptotic protein *Bax*, and effector protein *Cas3*. Table 3 shows relative gene expression values.

Table 3. Relative gene expression

	Control (O10T)	O1T	O2T	O3T	O4T	O5T	O6T	O7T	O8T	O9T	O11T	O12T
<i>Bax</i>	1	0.69	0.05	2.02	2.55	0.70	0.43	0.29	6.08	5.21	4.38	1.75
<i>Bcl-2</i>	1	1.05	2.64	1.87	1.48	1.33	0.88	1.64	1.67	3.01	2.25	1.72
<i>Cas3</i>	1	0.13	0.18	3.03	1.53	0.55	0.45	0.42	0.80	1.15	0.94	1.09

The obtained results show that relative gene expression for pro-apoptotic protein *Bax* and the anti-apoptotic protein *Bcl-2* is increased and that *Bax* predominates, which indicates the initiation of apoptosis, followed by a consequent increase in the relative expression of the gene for *Cas3*, as an effector caspase in apoptosis in samples O3T, O4T, O9T and O12T. In O8T and O11T samples, *Bax* and *Bcl-2* were elevated with a greater increase in *Bax*, but *Cas3* expression was slightly decreased. *Bcl-2* was elevated in samples O1T, O2T, O5T and O7T, while the expression of *Bax* was decreased, which indicates that apoptosis did not start in these samples.

This can also be seen in the relative expression of the gene for *Cas3*, which is quite reduced in these cases. In the O6T sample, both *Bax* and *Bcl-2* are decreased, but *Bcl-2* is less decreased than *Bax*, so anti-apoptotic mechanisms prevail in this sample, which is also indicated by the decreased relative expression of the gene for *Cas3* (Figure 1).

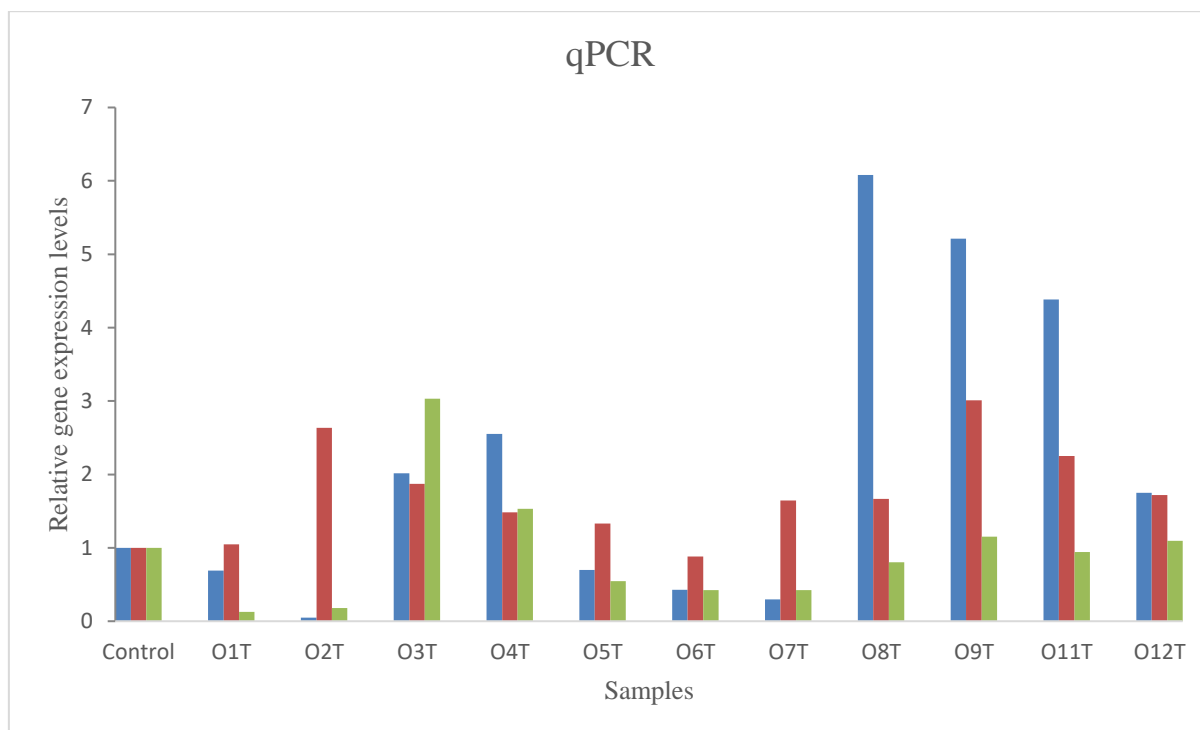


Figure 1. Quantitative polymerase chain reaction

DISCUSSION

Our study showed that it is possible to observe the process of apoptosis in ovarian benign, borderline, and malignant tumors by determining levels of expression of apoptotic-anti-apoptotic genes (*Bax*, *Bcl-2*) using quantitative PCR.

Although this analysis is of slight therapeutic importance because it is not used in standard clinical practice, further management of the disease is primarily based on histopathological examination and verification of the specimens and by further staging and grading of the tumor.

The B-cell leukaemia-2 gene (*Bcl-2*) is a proto-oncogene that inhibits apoptosis (VAUX *et al.*, 1988). In our study, the highest levels of expression were found in mucinous cystadenoma (O9T) and serous adenocarcinomas (O02T, O11T) meaning that in these tumors apoptosis was inhibited. Opposite, lowest levels of *Bcl-2* expression were found in benign (endometriotic cyst O6T, serous cystadenoma O5T) and borderline (mucinous O1T) tumors.

FAUVET *et al.* found a difference in *Bcl-2* expression among malignant, borderline, and benign groups of tumors. Lower *Bcl-2* expressions were found in borderline than in benign tumors (FAUVET *et al.*, 2005). These results conclude with ours. Furthermore, they found no difference in *Bcl-2* expression between benign, borderline, and malignant mucinous tumors, whereas there was a difference between borderline and benign serous tumors but not between borderline and malignant serous tumors. These results suggest the involvement of the mitochondrial apoptosis pathway in serous tumors. Their results are in line with ATHANASSIADOU *et al.* (1998) demonstrating that *Bcl-2* expression is cell and tissue dependent.

Bax is a pro-apoptotic protein. In our study, the highest levels of expression were in benign tumors (cystadenofibroma O8T, mucinous cystadenoma O9T) and one malignant tumor (serous adenocarcinoma O11T). The lowest *Bax* expression was found in benign tumors (endometriotic cysts O6T, O7T) and one malignant tumor (serous adenocarcinoma O2T).

FAUVET *et al.* (2005) observed *Bax* protein expression in borderline ovarian tumors, with no difference relative to benign tumors. Our results agree with theirs. A trend toward a difference was observed between malignant and borderline ovarian tumors (FAUVET *et al.*, 2005). Previous studies are in accordance with their results, showing no difference in *Bax* expression according to the histological type (WEHRLI *et al.*, 1998; MUNAKATA *et al.*, 2000). Moreover, WEHRLI *et al.* (1998) found no difference in *Bax* expression between malignant, borderline, and benign tumors in serous ovarian tumors. Altered genetic processes in cells lead to development of malignancies. It is considered that manipulation of these altered processes via gene therapy could lead to development of targeted therapy used for various malignancies, including ovarian (ROCCONI *et al.*, 2005).

CONCLUSION

Despite limitations of the current study including the sample size and the evaluation of limited apoptotic-antiapoptotic proteins, obtained results show that gene expression for pro-apoptotic protein *Bax* and anti-apoptotic protein *Bcl-2* is increased and that *Bax* predominates, which indicates the initiation of apoptosis, mostly in benign tumors. *Bcl-2* was elevated in samples of borderline and malignant tumors while the expression of *Bax* was decreased, which indicates that apoptosis did not start in these samples. Further studies are required to conclude whether the expression pattern of apoptotic-antiapoptotic proteins can be used as a clinical or histologic prognostic factor.

Ethics approval and consent to participate

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and 12 informed consents were obtained from the patients prior to enrollment in the study.

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