Human chorionic gonadotropin β subunit affects the expression of apoptosis-regulating factors in ovarian cancer

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Abstract. Expression of human chorionic gonadotropin, especially its free β subunit (hCGβ) were shown to play an important role in cancer growth, invasion and metastasis. It is postulated that hCGβ is one of the factors determining cancer cell survival. To test this hypothesis, we applied two models: an in vitro model of ovarian cancer using OVCAR-3 and SKOV-3 cell lines transfected with the CGB5 gene and an in vivo model of ovarian cancer tissues. The material was tested against changes in expression level of genes encoding factors involved in apoptosis: BCL2, BAX and BIRC5. Overexpression of hCGβ was found to cause a decrease in expression of the analyzed genes in the transfected cells compared with the control cells.

In ovarian cancer tissues, high expression of CGB was related to significantly lower BCL2 but higher BAX and BIRC5 transcript levels. Moreover, a low BCL2/BAX ratio, characteristic of advanced stages of ovarian cancer, was revealed. Since tumors were discriminated by a significantly lower LHCGR level than the level noted in healthy fallopian tubes and ovaries, it may be stated that the effect of hCGβ on changes in the expression of apoptosis-regulating agents observed in ovarian cancer is LHCGR-independent. The results of the study suggest that the biological effects evoked by hCGβ are related to apoptosis suppression.

Introduction

Human chorionic gonadotropin (hCG), which together with lutropin (LH), thyrotropin (TSH) and follitropin (FSH) belong to one glycoprotein hormone family, is produced during pregnancy by trophoblast cells (1). The hormone controls a number of processes, including embryo implantation, angiogenesis and development of the chorion (2).

The hormone is a glycoprotein composed of subunits α (hCGα) and β (hCGβ). Expression of human chorionic gonadotropin and in particular its β subunit (hCGβ) has been also documented for 30-60% of tumors of different origin (2,3). What is more, it has been shown that transcriptional activity of genes encoding hCG is higher in advanced cancers. Its presence in serum and urine of cancer patients and in tumor tissues in many cases is of prognostic significance as it correlates with a poor response to therapy and thus poor prognosis (4).

It is postulated that, similarly to the way chorionic gonadotropin promotes angiogenesis during placenta formation and modulates the mother’s immune system towards immunotolerance of the fetus, the hormone can play analogous functions in oncogenesis. This action of CG is said to contribute to neovascularization as well as desensitization of the immunological system towards cancer cells (5,6). Several publications also demonstrate that CG promotes tumor growth by exhibiting anti-apoptotic and/or proliferative effects (7,8).

Despite numerous studies on the role of CG and its free subunits in carcinogenesis, the biological functions and mechanisms behind their action remain unknown.

One hypothesis, which explains hCGβ action in cancers, is based on the hormone’s structural similarity to growth factors such as TGFβ (transforming growth factor β), PDGFβ (platelet-derived growth factor β) and NGF (nerve growth factor) characterized by the presence of a cysteine knot motif. It is suggested that due to this structural similarity, CG, like the aforementioned growth factors, may affect cells by regulation of their proliferation (9).

Recent studies have shown that the interaction of the β subunit of hCG with its receptor (LHCGR, luteinizing hormone/choriogonadotropin receptor) may lead to initiation of signaling cascades associated with extracellular signal-regulated kinases (ERK) and protein kinase B (PKB/Akt). The kinases are involved in cell cycle regulation, apoptosis and cancer pathogenesis (6,10). Thus, CG might also be considered a tumor-growth promoter.

Results of in vitro studies obtained independently by Hamada et al and our group support this hypothesis showing that silencing of CGB genes induces apoptosis (11,12). We demonstrated that the inhibition of CGB expression caused apoptosis both in cells expressing the anti-CGB construct as
well as in neighboring cells, lacking the construct. This neighboring cell effect could be explained by the overall decreased level of hCGβ in culture medium, a state which presumably allows more interactions between receptors and ligands essential for induction of apoptosis (12).

The in vitro results were verified on animal models. These experiments proved in turn that non-cancerous ovarian epithelial cells stably transfected with CGB overexpressed BCL-X(L) (B-cell lymphoma-extra large) and were characterized by a lower rate of apoptosis as well as more intense proliferation with increased levels of cyclin E/D1 and Cdk2/4/6. In consequence, cell phenotype changes led to increased tumorigenesis of xenografts in athymic nude mice. Histopathological analysis of tumors arising from implanted xenografts overexpressing hCGβ demonstrated that the cells were poorly differentiated (13).

What is more it has been shown that expression of hCGβ correlates with a decreased apoptosis rate in human cervical carcinomas (14). This suggests that the presence of hCG, produced by tumors, protects cells from initiation of apoptosis, allowing tumor development and growth.

Thus, in the present study we attempted to verify the association between expression of CGB and factors regulating apoptosis such as BCL2 (B-cell lymphoma 2), BAX (Bcl-2-like protein 4) and BIRC5 (survivin).

Materials and methods

Specimens of ovarian cancer tissue were obtained from 45 patients with ovarian cancer treated by surgery at the Department of Gynecologic Oncology, Poznan University of Medical Sciences. Histological subtypes and the grade of the carcinomas are presented in Table I.

The control group included samples of ovaries (n=8) and fallopian tubes (n=6) that lacked cancerous transformations as assessed by macroscopic and microscopic examination by a pathologist. Control samples were obtained from post-menopausal patients who underwent total hysterectomy with additional oophorectomy due to myomas.

Both ovarian cancer tissues and control tissues were maintained in RNAlater buffer (Sigma Life Sciences, St. Louis, MO, USA) at -80˚C prior to further processing.

The study was approved by the Ethics Review Board of Poznan University of Medical Sciences (resolution no. 748/08) and all patients participated after informed consent.

SKOV-3 and OVCAR-3 cell lines, established from ovarian carcinomas and used as an in vitro model of cancer, were obtained from the Global Bioresource Center, American Type Culture Collection (ATCC, Manassa, VA, USA; SKOV-3 ATCC® HTB-77™, OVCAR-3 ATCC® TB-161™). The choice of cell lines was determined by the fact that SKOV-3 is characterized by the lack of LHCGR expression, while OVCAR-3 cells express the receptor (15). The presence and lack of LHCGR expression in the analyzed cells was also validated experimentally by quantitative polymerase chain reaction (qPCR) (data not shown). Cells were cultured and passaged under standard conditions.

Cell culture and transfection. Cells were seeded so as to at the time of transfection obtain the optimal 70-80% confluence. Transfection with the construct carrying the CGB5 gene, chosen as one of the most transcriptionally active CGB genes (17), utilizing TurboFect (Thermo Scientific, Rockford, IL, USA) was performed according to the manufacturer's protocol. The CGB5 construct was prepared as described previously (16).

Non-transfected cells were used as control. Additionally in order to estimate the transfection efficiency, an equal amount of reporter eGFP plasmid (Invitrogen, San Diego, CA, USA) was used to transfect the study cell lines.

Analyses were conducted 72 h after transfection. All experiments were performed in triplicates.

RNA isolation. Total RNA from the SKOV-3 and OVCAR-3 cells as well as from the analyzed tissues (100-300 mg) was isolated using TriPure Isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. An air-dried pellet of RNA was suspended in UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA USA). RNA was stored at -80˚C prior to subsequent steps.

cDNA synthesis. One microgram of RNA was used for each reverse transcription reaction with universal primer p(dT)10 and Transcriptor Reverse Transcriptase (Roche Diagnostics), according to the delivered protocol.

qPCR. To assess the expression level of the analyzed genes, qPCR with sequence-specific primers, hydrolysis probes and the LightCycler® TaqMan® Master kit (Roche Diagnostics) was performed. Probes and primers used in the reactions are presented in Table II.

The reaction mix for the TaqMan reactions contained: 5 µl of cDNA, 1X TaqMan Master Mix (Roche Diagnostics), 0.1 µM hydrolysis probe (TaqMan) and 0.4 µM of each primer. qPCR program consisted of initial denaturation at 95˚C for 10 min followed by 45 3-step cycles: 95˚C/10 sec hold for denaturation, 60˚C/30 sec hold for primers and probe hybridization and product extension, and 72˚C/1 sec hold for data acquisition.

Relative expression of genes analyzed by TaqMan assays was normalized against HPRT expression (Human HPRT Gene Assay; Roche Diagnostics). All experiments were performed in triplicates using independently synthesized cDNA.

<table>
<thead>
<tr>
<th>Ovarian carcinoma subtype</th>
<th>Tumor grade</th>
<th>Serous</th>
<th>Endometrial</th>
<th>Mucinous</th>
<th>Clear cell</th>
</tr>
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<tbody>
<tr>
<td>G1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>22</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Not determined</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Total</td>
<td>34</td>
<td>8</td>
<td>2</td>
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</tr>
</tbody>
</table>

Table I. The histological subtype and grade of the studied ovarian carcinomas.
Mann-Whitney U test in case of cell lines and Kruskal-Wallis software package (StatSoft, Krakow, Poland) with Data were analyzed using the Statistica 10 Statistical analysis.

Cell lines, OVCAR-3 and SKOV-3, were transfected with them against the induction of apoptosis, ovarian cancer (non-transfected) cells, respectively. Differences for both >3-fold lower (p<0.01); transfected cells were characterized by an average >4-fold lower. In the SKOV-3 cells, significant differences were noted for BAX expression only (p<0.01), where in the control cells the gene expression was on average >4-fold lower.

No significant differences in the BCL2 to BAX expression ratio were observed between the control and transfected cell (Fig. 2C and Fig. 3C).

Similarly to BCL2 and BAX, expression level of the BIRC5 gene encoding survivin was noted to decline in the transfected cells (Fig. 2A and B and Fig. 3A and B). In the case of the transfected OVCAR-3 cells, expression of BCL2 and BAX was on average 2-fold lower than that in the control cells; differences were statistically significant (p=0.05 and 0.05, respectively). In the SKOV-3 cells, significant differences were noted for BAX expression only (p<0.01), where in the control cells the gene expression was on average >4-fold lower.

Overexpression of CGB affects the expression of BCL2, BAX and BIRC5 in the ovarian cancer cell lines. In order to test the hypothesis that hCGβ synthesized by cancer cells protects them against the induction of apoptosis, ovarian cancer cell lines, OVCAR-3 and SKOV-3, were transfected with a construct carrying the CGB5 gene, which is one of the most transcriptionally active CGB genes (17).

Introduction of the construct into OVCAR-3 and SKOV-3 cells resulted on average in 80% efficiency of transfection and thus a profound overexpression of CGβ subunit mRNA in both cell lines. The relative level of CGB1-9 transcripts in the transfected OVCAR-3 and SKOV-3 cells was on average >3,000- and 15,000-fold higher than that in the control (non-transfected) cells, respectively. Differences for both cell lines were statistically significant (p<0.001 and p<0.001, respectively, Fig. 1).

Analysis of BCL2 and BAX expression in the cell lines overexpressing CGB5 showed that the mRNA level of these agents declined in the transfected cells (Fig. 2A and B and Fig. 3A and B). In the case of the transfected OVCAR-3 cells, expression of BCL2 and BAX was on average 2-fold lower than that in the control cells; differences were statistically significant (p=0.05 and 0.05, respectively). In the SKOV-3 cells, significant differences were noted for BAX expression only (p<0.01), where in the control cells the gene expression was on average >4-fold lower.

No significant differences in the BCL2 to BAX expression ratio were observed between the control and transfected cell (Fig. 2C and Fig. 3C).

Similarly to BCL2 and BAX, expression level of the BIRC5 gene encoding survivin was noted to decline in the transfected cells (Fig. 2D and Fig. 3D). Statistically significant differences were noted between the control and transfected SKOV-3 cells (p<0.01); transfected cells were characterized by an average >3-fold lower BIRC5 expression than the control cells.

Since the physiological effect of CG is mediated via the receptor for luteinizing hormone and hCG, transfected cells were analyzed in terms of the receptor expression. In the case of OVCAR-3 cells, CGB5 overexpression was associated with an almost 2-fold decrease in the relative level of LHCGR mRNA, but the difference was not statistically significant (Fig. 4). Increased expression of CGB5 in the SKOV-3 cells, lacking LHCGR, did not influence the expression of the receptor; no LHCGR expression was noted (data not shown).

All analyses were made on the basis of three independent experiments and from each three independent experiments, cDNA was used for qPCR.

Table II. Primers and hydrolysis probes used in qPCR assays.

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Starters and probes</th>
</tr>
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<tbody>
<tr>
<td>CGB1-9</td>
<td>5'-TACTGCCCCACCATGACC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CACGCCCTAGGACAC-3'</td>
</tr>
<tr>
<td></td>
<td>#71 (Roche Diagnostics UPL)</td>
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<tr>
<td>LHCGR</td>
<td>5'-CAGCTCAGTATCATTGCTACTTC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GAAAGAATTTTACCTGTAATTTG-3'</td>
</tr>
<tr>
<td></td>
<td>6FAM-CAGGCATGAAAGTTTCCAGATGTACGA-BBQ</td>
</tr>
<tr>
<td></td>
<td>(TIB MOLBIOL)</td>
</tr>
<tr>
<td>BAX</td>
<td>5'-ATGTTTCTGACGGCAACTTC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ATCACCCGGCCACCTTG-3'</td>
</tr>
<tr>
<td></td>
<td>#57 (Roche Diagnostics UPL)</td>
</tr>
<tr>
<td>BCL2</td>
<td>5'-TACCTGAAACGGCACTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCCGTACAGTTCCACAAAGG-3'</td>
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<tr>
<td></td>
<td>#75 (Roche Diagnostics UPL)</td>
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<tr>
<td>BIRC5</td>
<td>5'-TCTGCTTCAAGGAAGCTGGA-3'</td>
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<tr>
<td></td>
<td>5'-AAAGTGCTGATTACAGCGTA-3'</td>
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<td></td>
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<td>(Roche Diagnostics UPL)</td>
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</table>

Statistical analysis. Data were analyzed using the Statistica 10 software package (StatSoft, Krakow, Poland) with Mann-Whitney U test in case of cell lines and Kruskal-Wallis test with Dunn's post hoc test in case of tissues. Differences for both >3,000- and 15,000-fold higher than that in the control (non-transfected) cells, respectively. Differences for both

![Figure 1. Relative expression levels of CGB1-9 genes in OVCAR-3 (A) and SKOV-3 (B) cells after transfection with the construct carrying CGB5.](image-url)
Ovarian cancer is characterized by altered expression of the BCL2, BAX and BIRC5 genes. The experiments conducted using RNA isolated from ovarian cancers, fallopian tubes and healthy ovaries showed that all analyzed tissues expressed...
CGB1-9 and the level of gene expression differed between the groups (Fig. 5).

Fallopian tube tissues were characterized by lower CGB1-9 expression than the other analyzed groups. On average it was ~60,000- and 40,000-fold lower than the level in the healthy ovaries and ovarian cancer, respectively. Thus, statistically significant differences were observed between the group of fallopian tubes and healthy ovaries (p<0.05) as well as between fallopian tubes and ovarian cancers (p<0.01).

Assessment of the BCL2 gene, encoding an anti-apoptotic factor, showed that ovarian cancers had a statistically significantly lower BCL2 expression level than the fallopian tube (p<0.001) and healthy ovary tissues (p<0.01, Fig. 6A) and that it was on average almost 30- and 10-fold lower, respectively. An opposite phenomenon was observed in case of the pro-apoptotic BAX gene analysis. Evaluation of BAX expression showed that the group of ovarian cancer tissues was characterized by distinctly higher amount of the gene transcripts than the fallopian tubes and healthy ovaries (Fig. 6B). On average it was almost 50- and >100-fold higher, respectively, and the differences proved to be statistically significant (p<0.01 and p<0.001, respectively).

Consequently a decrease in the BCL2 to BAX expression ratio was reported (Fig. 6C). Differences in the BCL2/BAX between the ovarian cancer and fallopian tube tissues as well as the ovarian cancers and healthy ovaries were statistically significant (p<0.001 and p<0.0001, respectively).

The BIRC5 gene, coding for yet another factor regulating apoptosis, was shown to have on average a >30-fold higher expression level in case of the ovarian cancer than the level in the fallopian tube and healthy ovarian tissues. Differences in BIRC5 expression between the groups were also found to be statistically significant (p<0.01 and p<0.001, respectively, Fig. 6D).

In all studied tissues, the expression level of the receptor for luteinizing hormone and human chorionic gonadotropin was also evaluated. The results confirmed that not all analyzed samples expressed LHCGR at the RNA level. In 13 out of 45 ovarian cancer tissue samples, LHCGR expression was found to be below the adopted qPCR assay’s detection range. In these cases zero was replaced with a value 10% lower than the lowest LHCGR expression level observed in the assay. This allowed performing full statistical analysis, which proved that again the ovarian cancer tissue expression profile was different from the profiles of the fallopian tube and healthy ovarian tissues. The ovarian cancer tissue group was discriminated by a lower LHCGR expression level than the level in the other two analyzed groups. It was especially distinct between cancers and fallopian tubes as it was on average >10-fold lower. Differences in the gene expression between ovarian cancer and fallopian tube tissues as well as ovarian cancer and healthy ovarian tissues were shown to be statistically significant (p<0.001 and p<0.05, respectively, Fig. 7).

Spearman’s rank order correlation analysis pointed to statistically significant relations within the three studied tissue groups. Among the cancer tissue cases, the relative level of LHCGR transcripts was negatively correlated with the expression level of BIRC5 encoding survivin (p<0.05; R=-0.39).

On the other hand, in the group of fallopian tube tissues, LHCGR expression level showed a very strong positive correlation with the number of BCL2 transcripts (p<0.05; R=0.94).

The highest number of statistically significant correlations was however noted for the group of healthy ovaries. In these tissues, CGB expression showed a very strong positive correlation with survivin expression (p<0.05; R=0.80) as well as a strong negative correlation with transcriptional activity of LHCGR (p<0.05; R=-0.74). In this group, BAX expression was also correlated positively with the BCL2 expression level (p<0.05; R=0.74), while LHCGR was correlated negatively with the survivin mRNA level (p<0.05; R=-0.79).

All analyses were carried out based on the results obtained in qPCR with the use of three independent cDNAs synthesized for each RNA sample.

Discussion

Production of CG and its free β subunit by tumors of different origins is a well-known phenomenon (4). However, the mechanism by which these interchangeable cancer promoters achieve their biological effects is not fully understood. The two most often pursued hypotheses concern direct influence on cancer cell proliferation or/and indirect cancer cell survival promotion by inhibiting apoptosis signals (7,11,12,18).

The results of our previous studies as well as data published by Hamada’s group demonstrated that silencing of the chorionic gonadotropin β subunit leads to induction of
programmed cell death in cancer cells cultured in vitro (11,12). What is more it was shown that the expression of hCGβ was correlated with a decreased apoptosis rate in human cervical carcinoma samples analyzed by IHC (14). This suggests that hCG produced by tumors may protect cells from initiation of apoptosis, allowing tumor development and growth.

In the present study, the expression level of BCL2, BAX, and BIRC5 genes involved in the apoptosis of ovarian cancers expressing CGB was analyzed. Selection of these factors was based on the fact that they are key players in the activation of caspases in the final stages of apoptosis. Moreover, their aberrant expression marks a variety of tumors (19).

In the first step of the study, the influence of hCGβ on the expression of BCL2, BAX and BIRC5 in an in vitro model was examined. Introduction of the construct carrying the CGB5 gene into OVCAR-3 and SKOV-3 cells resulted in a profound, statistically significant increase in CGB expression in both cell lines.

Overexpression of the hormone’s β subunit caused a decrease in BCL2 and BAX transcript level in both transfected cell lines. However no significant differences were observed in the BCL2 to BAX expression ratio between the transfected and control cells.

Similarly to BCL2 and BAX, the expression of the BIRC5 gene encoding survivin was also noted to decline in the transected cells compared to the control (non-transfected) cells.

The same analyses were conducted on RNA isolated from the ovarian cancer tissues. Ovarian cancer can be very heterogeneous at both the histological and genetic level. It was suggested recently that the source of both low- and high-grade serous carcinoma is the fallopian tube epithelium (benign or malignant) implanted on the ovary, rather than the ovarian surface epithelium itself, as previously believed (20). In the view of this new model of ovarian tumorigenesis, the control groups of our study comprised healthy ovaries and fallopian tubes.

All studied tissues, both cancer and healthy, were characterized by the presence of CGB transcripts. However, the relative expression level of the hormone’s subunit varied. Fallopian tube tissues were distinguished by a lower CGB1-9 expression level than that in the other analyzed tissues. Statistically significant differences were observed between the group of

Figure 6. Relative expression level of BCL2 (A), BAX (B), BIRC5 (D) and the BCL2/BAX ratio (C) in the studied tissues: fallopian tubes, healthy ovaries and ovarian cancer.

Figure 7. Relative expression level of the LHCGR gene in the studied tissues: fallopian tubes, healthy ovaries and ovarian cancer.
survivin in tumors, similarly to CGB, is often associated with an invasive phenotype and resistance to chemotherapy and radiotherapy and consequently with poor prognosis (27).

In both cell lines CGB overexpression led to a decrease in all analyzed apoptosis-related factors, while in the ovarian cancer tissues CGB expression was associated with an increase in BAX and BIRC5 expression. The reason why the results of our in vivo studies did not completely match the results of the in vitro study may be related to the fact that the in vitro model did not completely reflect the complexity of the tissue. Therefore, it must be taken into consideration that the final effect of hCGβ action in tissues depends on the interaction between different cell types and responses induced by the hormone.

CG acts via the receptor for luteinizing hormone and human chorionic gonadotropin, thus the expression level of LHGR in the studied cell lines and tissues was evaluated. SKOV-3 cells lack LHGR, and overexpression of CGB did not influence LHGR transcriptional activity. On the other hand, in the transfected OVCAR-3 cells, a decrease in the amount of LHGR mRNA was observed, however differences were not statistically significant.

These results were confirmed also by the analysis performed on RNA isolated from cancer tissues. In accordance with other studies showing that not all tumors with confirmed hCGβ expression were characterized by the presence of LHGR (12), only part of the analyzed samples expressed LHGR. In fact, in 13 out of 45 ovarian cancer cases, LHGR was not detected. What is more, ovarian cancers were discriminated by a significantly lower LHGR expression level than the one noted for healthy ovarian and fallopian tube tissues.

Thus, both in vitro and in vivo studies suggested that CGB expression in cancer cells regulates LHGR expression. Negative LHGR regulation under CG and hCGβ influence has been previously reported at both the mRNA and protein levels (17,29). Our data together with the fact that hCGβ affects SKOV-3 cells lacking the receptor, suggests that the effect of the hormone on cancer cells is LHGR-independent.

In the present study, the analyzed tumor tissues consisted of ovarian cancers, most of which, according to the newest classification, belonged to type II tumors (present in advanced stage and comprising high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors, carcinomas, and undifferentiated carcinomas) (19). Since the analyzed cancer tissues were a rather homogenous group, no attempt was made to correlate the obtained results with clinical data.

The correlations we found linked CGB positively with BIRC5 and negatively with LHGR expression in healthy ovaries. Within this tissue group, strong positive correlation between BAX and BCL2 expression was also found. Surprisingly a very strong positive correlation between LHGR and BCL2 in the fallopian tube group was noted while in cancer LHGR expression was negatively related with BIRC5. The meaning of these correlations is not clear.

In conclusion, even though the exact molecular mechanism of hCGβ action in cancer cells still needs to be established, the results of the present study together with the fact that tumors expressing the free β subunit of hCG are characterized by a more malignant phenotype and worse prognosis, suggest that the biological effect of hCGβ is related to suppression of
apoptosis. Protection of tumor cells from programmed cell death induction may be achieved by expression modulation of genes regulating apoptosis such as BCL2, and BAX and BIRC5.

Acknowledgements

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References