Isomorph Expression of *BAG-1* Gene, ER and PR in Endometrial Cancer

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Abstract. *Background: BAG-1 isomorphs are regulating proteins with antiapoptotic action in endometrium. ERa and PRA isomorphs seem to have an important role in endometrial cancer. Patients and Methods: We investigated the expression of BAG-1, ERa and PRA isomorphs in endometrioid adenocarcinoma and we correlated them with clinicopathological findings of the tumor. Fresh endometrial tissues were obtained from 33 patients with endometrial carcinoma and 191 paraffin-embedded tissues were analyzed by real-time PCR and immunochemistry for BAG-1, ER and PR. Results: BAG-1 protein is expressed in both nucleus and cytoplasm. Grade 3 tumors were considered to have the highest intensity. Only 4 out of 79 samples showed intense expression of ERa, while 37 samples out of 72 samples strongly expressed PRA. Conclusion: BAG-1 nuclear isomorph appeared more frequently in grade 2 tumors than in grade 1 and 3 tumors, and the cytoplasmatic isomorph was expressed more strongly than the nuclear one.*

Programmed cellular death, so-called apoptosis, is a homeostatic mechanism in multicellular organisms regulated by a large number of various proteins (1). BAG-1 isomorphs are among such regulating proteins, having antiapoptotic action (2). These isomorphs result from the alternating maturation of a single mRNA at the translation level (3).

BAG-1S (p36, 36 kDa), located predominantly in the cytoplasm, and BAG-1L (p50, 50 kDa), which is found mainly in the nucleus, are two of the isomorphs (4, 5). These isomorphs interact with a variety of cellular targets and regulate important control pathways in both normal and malignant cells (4). The *BAG-1* gene has been found to be overexpressed in quite a few tumor types, *e.g.* in prostate, cervical and stomach cancer (7, 8). Recently, it was recognized as a new prognostic marker for the survival of patients with early-stage breast cancer (9) and its action seems to be connected to the expression of estrogen (ER) and progesterone receptors (PR).

In quite a lot of mammals, steroid hormones seem to regulate apoptotic mechanisms in endometrium (7). Indeed, the use of estrogens for long-lasting periods seems to be related to an increased risk of developing endometrial cancer in humans (10). Among ERs, ERα is the predominant one, which is usually found in normal endometrium during the menstrual cycle (11), in endometrioid carcinomas (12) and in endometrioid tissues (13).

Regarding PRs, there are two known isomorphs, A and B. Progesterone is their natural binder, causing their homodimerism and heterodimerism. Studies have shown that PR-A is required for progesterone to act on the reproductive system, including uterus (14).

The aim of our study was to investigate the expression of BAG-1, ERα and PR-A isomorphs in endometrioid adenocarcinoma and to correlate them with clinicopathological findings such as grade and stage of the tumor.

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Patients and Methods

Study population and specimen collection. Fresh endometrial tissues were obtained from 33 patients with endometrial carcinoma (30 with endometrioid adenocarcinoma, 1 with clear-cell carcinoma and 2 with serous-papillary carcinoma) and 191 paraffin-embedded tissues from patients who had received abdominal hysterectomies at the First Gynecological Clinic of the General Anticancer Hospital of Athens (Greece) from 1986-2007. All patients were Caucasians with an age range of 32-82 years (mean 63.3 years).

The classification of endometrial phase was according to the criteria of Noyes and Hertig (15). Each specimen was reviewed by two expert pathologists and classified into groups according to the FIGO grading system (15) (Table I). Histological differentiation of endometrial carcinomas was divided into well-differentiated, composed of glands (G1, n=11); moderately differentiated, a combination of glands and masses of solid epithelium (G2, n=7); and poorly differentiated, predominantly solid proliferations (G3, n=4) (16). Our samples were categorized according to the clinical staging into three major groups: first group includes stage Ia; second group includes stages Ib, Ic and IIa; and third group IIb, III and IV. Twenty-three out of 186 samples belong to the first group, and 119 and 30 to the second and third, respectively. Tumor characteristics are given in Table I.

Table I. *Clinicopathological data, complementary therapy and recurrence*.

Variable	No. of cases
Histological cell type	
Endometrioid	144
Serous-papillary	7
Clear cell	2
Adenosquamous	2
FIGO stage	
Ia	23
Ib + Ic + IIa	119
IIb + III + IV	30
FIGO grade	
1	64
2	34
3	61
Lymph-node excision	
No	84
Yes	60
Unknown	2
Lymph-node metastasis	
Negative	53
Positive	7
Peritoneal cytology	13
No washings	118
Negative	9
Positive	8
Unknown	79
Follow-up therapy	
External radiotherapy (RT)	37
External RT + brachytherapy	21
Brachytherapy	4
Chemotherapy	3
External RT + hormonotherapy	1
Hormonotherapy	2
Recurrence	
No	9
Yes	14
Recurrence site	
Vagina	4
Pelvis	3
Abdomen/intestine	3
Distant	4

RNA extraction and cDNA synthesis. As previously described (17), total cellular RNA was obtained from tissue biopsies. Five micrograms of RNA per sample were separated on 1% formaldehyde-agarose gels to assess RNA integrity. RNA concentrations were qualified by spectrophotometry (E=260 nm). First-strand cDNA was synthesized from 4 μg of total RNA, using an Oligo(dT)12-18 primer and the SuperscriptTM II RNase Reverse Transcriptase (Invitrogen, USA). Samples were stored at –20˚C.

Real-time PCR. Thirty-three biopsies were processed for real-time PCR analysis. The quantification of the selected genes by real-time PCR were run using Rotor Gene 6000 the series software version 1.7 (Corbett Life Science, USA). The reaction mixture consisted of 2 μl cDNA, 1 μl of each primer (400 nM) and 21 μl reaction buffers (Platinum SYBR Green) (total reaction volume 25 μl) (Invitrogen, USA). Real-time PCR cycles consisted of: 2 minutes at 50˚C, 4 minutes at 95˚C for polymerase activation, 45 cycles of 10 seconds at 95˚C (denaturation), 5 seconds at 54˚C, 5 seconds at 72˚C and 15 seconds at 83˚C (annealing and extension). Finally, melting occurred at 72˚C-95˚C (0.5˚C increase) for 5 seconds for each step. β-actin of each sample served as intrinsic control. The threshold cycle (CT) of each sample was normalized to the β-actin. A relative quantification analysis was carried out with series software version 1.7 Rotor Gene 6000. The analysis uses the sample's crossing point, the efficiency of the reaction, the number of cycles completed and other values to compare the samples and generate the ratios. The results are expressed as a normalized ratio.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissues were used for this study. Samples representing hysterectomies were fixed in 4% buffered formalin and then routinely processed for paraffin embedding. Five-micrometer-thick sections were obtained by microtome. They were dried in an autoclave at 60˚C for 30 minutes. Trilogy solution (Cellmarque, USA) was used for deparaffinization, rehydration and unmasking. The samples were then placed in Perox Free Block solution (Cellmarque, USA) for 10 minutes to inhibit endogenous peroxidases then in EnVision and Dual Link System Peroxidase (DakoCytomation, USA) for 25 minutes. The antibodies that were used for the detection of BAG-1, ERα and PR-A were mouse anti-human monoclonal antibodies Clone: SPM232, (Spring Bioscience, USA), Clone: 1d5 (DakoCytomation, USA) and Clone: PgR636 (DakoCytomation, USA), respectively. Liquid DAB and the Substrate Chromogen System (DakoCytomation, USA) was used for 6 minutes as chromogen.

Staining evaluation and statistical analysis. The stained slides were microscopically analyzed independently by two expert pathologists. The results of immunostaining for BCL-2 and BAX were analyzed

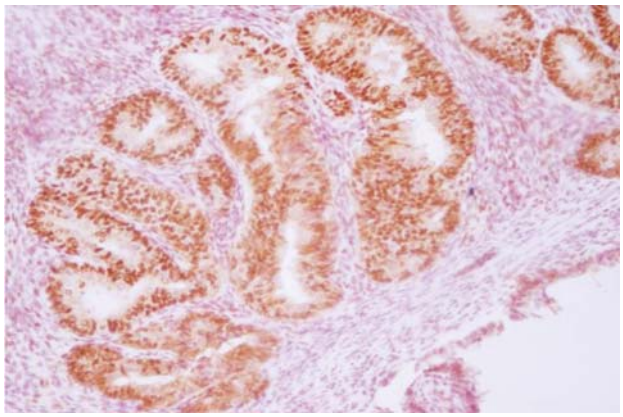

Figure 1. *Nuclear immunohistochemical stain in endometrioid adenocarcinoma of the endometrium for the ERa antigen-2 and 3M intensity (×400) (Nikon HFX-IIA).*

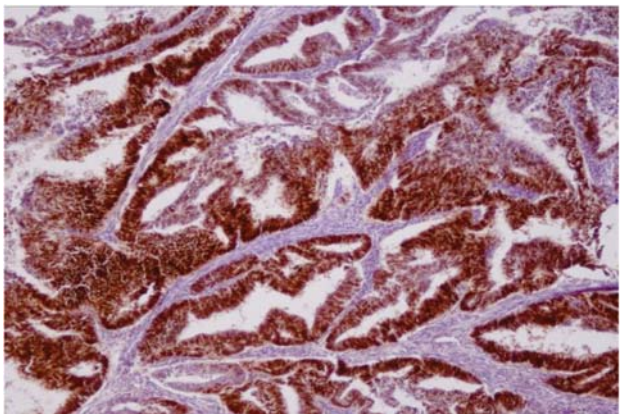

Figure 2. *Nuclear immunohistochemical stain in endometrial adenocarcinoma of the endometrium for the PR-A antigen, 3M intensity (×400) (Nikon HFX-IIA).*

semi - quantitatively by using an immunohistochemical histological score (HSCORE), which incorporated both the intensity and the distribution of specific staining. The HSCORE was formulated as HS = Σ (Pi × i/100), where Pi denotes the percentage of stained cells and I denotes the intensity of staining which ranges from 1 to 3. The immunostaining for p53 was evaluated as 0 for negative tissues, 1 for 0-10% p53-positive cells, 2 for <50% positive cells and 3 for >50% positive cells.

Data were assessed with analysis of variance (ANOVA) and Chi-square with SPSS software version 13.0 (USA). Statistical significant was accepted at $p<0.05$.

Results

The immunohistochemical analysis showed that BAG-1 protein is expressed in both nucleus and cytoplasm. Staining intensity was measured and grade 3 considered to be the highest intensity. Out of 164 samples, 58 showed intense expression (grade 3) in both cytoplasm and nucleus, 19 samples showed moderately intense staining (grade 2) and 27 samples weak expression (grade 1). Finally, 17 samples failed to express the protein in either the nucleus or the cytoplasm. Moreover, in the majority of samples (51/79), the cytoplasmatic isomorph was expressed more strongly than the nuclear one.

Regarding Erα (Figure 1), based on the HSCORE, only 4 out of 79 samples showed intense expression, 23 expressed it moderately and 52 were negative. Regarding PR-A expression (Figure 2), in 37 samples out of 72 samples it was strongly expressed, in 14 moderately and 21 were negative.

Concerning *BAG-1* gene, there was a statistically significant ($p<0.013$) but slight correlation for its nuclear isomorph to be found strongly expressed in moderately differentiated tumors (grade 2). Statistical analysis revealed no correlation for isomorph expression to either ER or PR. Additionally, the observation that BAG-1 isomorph was also being expressed outside neoplastic cells was further examined. We found a high correlation between isomorph expression and its location outside neoplastic cells, in endometrial stroma and/or vessels, which is statistically significant.

Finally, the absence of expression of PR (HSCORE=0-0.05) was found to be slightly correlated to poorly differentiated tumors (grade 3) in a statistically significant way. On the other hand, no statistically significant correlation was noted between the expression of PR-A or ERα and the clinical staging of the neoplasm.

Discussion

Hormonal level changes, and in particular those concerning steroids, impact the apoptotic mechanism, which varies morphologically and biochemically during the menstrual cycle (17). Isomorphs of *BAG-1* gene are overexpressed in quite a few tumors, *e.g.* prostatic and cervical neoplasms (6, 7, 18, 19), possibly having an important role in their growth. This is based on the fact that its overexpression suspends cellular death, *e.g.* in stomach cancer (20), while changes in its expression have been observed in preinfiltrative breast diseases (21). Our study revealed that the cytoplasmatic form prevailed over the nuclear in endometrioid cancer. Song *et al.* (22) observed that the expression of cytoplasmatic isomorph is more frequent in neoplastic endometrial cells than in normal cells, while the nuclear isomorph is expressed more often in normal cells.

BAG-1 nuclear isomorph was found more frequently in moderately differentiated tumors (grade 2), and this was statistically significant. In quite a few papers, researchers

have suggested that overexpression of BAG-1 isomorphs, both cytoplasmatic and nuclear, as in breast cancer (9, 23, 24), is correlated to tumor clinicopathological features (9, 25, 26, 28). In particular, increased levels of cytoplasmatic isomorph expression are related to higher survival rates in patients who receive hormonal therapy. Unfortunately, the small number of patients treated with hormones does not permit statistical analysis and correlation of hormones to BAG-1 isomorph protein expression. Tang *et al*. (24) noted that increased expression of the nuclear isomorph was linked to poor outcome, as in cases of breast and laryngeal cancer (27), in contrast with other scientists (9). Indeed, neoplasms of moderate or poor differentiation are more likely to express both nuclear and cytoplasmatic isomorphs simultaneously (26).

Our results, regarding BAG-1, ER and PR expression, in which there was no statistically significant correlation observed, are in agreement with certain studies (24) but not with others, which correlate patient's successful response to hormone therapy with protein expression, *e.g*. in breast cancer (6) but also in early stages of endometrial cancer (26). Indeed, some researchers (26) mentioned that abnormal hormonal levels may act as a catalyst giving rise to various types of endometrioid cancer. Long-term elevating of estrogen levels without progesterone inhibitory effect are thought to lead to abnormal cell proliferation in the endometrium. In studies of breast cancer, a direct correlation between BAG-1 and functional ER has been proposed. Cutress *et al*. mention the role of BAG-1L as an important ER regulator. More specifically, it reinforces estrogen (136+137) transcriptase response. Regarding PR expression, its nuclear isomorph has been linked to a significantly higher survival rate in patients who receive hormonal therapy (8, 28).

It should be stressed here that besides endometrium, BAG-1 protein expression was also observed in adjacent tissues, specifically in endometrial stroma and vessels. In fact, our results showed that these proteins are expressed either in endometrial stromal cells only or in stromatic and endothelial vessel cells, rather than just in the vessels.

Regarding endometrium, most studies refer to ERα activation mechanisms, while there is little information about ERβ (29). PR has also been implicated in endometrial neoplasms development. Progesterone cellular impact is determined by its two receptors, PR-A and -B, while reduced expression of PR-B has been observed in poorly differentiated tumors in endometrial cancer, cell lines and certain breast cancers (30, 31).

ERα is also remarkably decreased in endometrium adenocarcinomas, as well as in other neoplasms, *e.g*. in ovary and colon, where the main receptor expressed is ERβ (32). In some studies, a correlation has been noted between ER expression and histological grade of differentiation and clinical staging (33), while our results did not show any correlation with these parameters.

ER and PR are strongly linked to survival, while PR expression seems to be more reliable for predicting survival in cases of endometrioid adenocarcinomas compared to ER expression, regardless of other clinicopathological parameters (34). Certain researchers stress the lack of ER in patients with poor prognosis (35), mainly in those with metastatic tumors who had previously undergone hormonal therapy (36). Both ERα and PR-A expression is related to histological subtype and grade of histological differentiation but there was correlation to survival (37). Our results showed a statistically significant absence of PR-A expression in tumors with poor differentiation (grade 3). In the literature, ER and PR expression is known to be frequent in well-differentiated endometrioid adenocarcinomas (38), but there is little information regarding the hormonal profile in clear-cell carcinomas (39, 40) and carcinosarcomas (38).

In recent years, an increasing number of studies have been carried out on endometrial hormone receptor expression, with many researchers suggesting that they may constitute an important pharmaceutical target for endocrine treatment of endometrial cancer (4). The coexistence of hormone receptors in tumors with poor and moderate differentiation demonstrates the impact of endogenous estrogen and progesterone hormones. This should be further studied because the possible benefit of hormonal therapy to endometrial cancer patients could be crucial as regards disease progression (35). Estrogen therapy has already been mentioned (35), but progesterone therapeutic results are not yet completely known.

The objective of the present study was to highlight possible changes in the expression of these important molecular regulators of the apoptotic mechanism and their correlation with clinicopathological findings.

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