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Hormone receptor expression profile of low-grade serous ovarian cancers

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HIGHLIGHTS

- We describe the hormone receptor profile of low-grade serous ovarian cancer, LGSOC.
- We show that LGSOCs express ERα, ERβ isoforms (ERβ1, ERβ2 and ERβ5), PR and AR.
- Cytoplasmic ERβ2 is high in metastases, in line with an anti-apoptotic role.
- Nuclear ERβ1 is high in borderline tumors, in line with an antitumoral role.
- Overall our findings suggest a key role for ERβ signaling in LGSOC development.

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ABSTRACT

Objective. Low-grade serous ovarian carcinomas (LGSOCs) are a histological subtype of epithelial ovarian tumors, accounting for fewer than 5% of all cases of ovarian carcinoma. Due to the chemoresistant nature of this subtype a search for more effective systemic therapies is actively ongoing, hormonal therapy showing some degree of activity in this clinical setting. The present study ought to investigate the hormone receptor status of LGSOCs, as a strategy to provide molecular support for patient-tailored hormonal treatments.

Methods. Estrogen receptor α (ERα), ERβ isoforms (i.e. ERβ1, ERβ2 and ERβ5), progesterone and androgen receptor (PR, AR) expression was evaluated by immunohistochemistry in 25 untreated LGSOC primary tumors, 6 matched metastases and 6 micropapillary variant of serous borderline tumors (micropapillary SBOTs). *In vitro* cellular models were used to provide insights into clinical observations.

Results. Our results showed prominent expression of nuclear ERα, ERβ2, ERβ5 and PR in LGSOC primary tissues, while metastatic lesions also exhibit considerable cytoplasmic ERβ2 levels. Notably, a higher expression of ERβ1 protein was determined in micropapillary SBOTs compared to LGSOCs. In vitro experiments on LGSOC cell lines (i.e. HOC-7 and VOA-1056) revealed low/absent ERα, PR and AR protein expression, whereas the three ERβ isoforms were all present. Proliferation of HOC-7 and VOA-1056 was not modulated by either the endogenous or the selective synthetic ligands.

Conclusions. These novel findings highlight the need of assessing relative levels of ERα and ERβ isoforms in the total receptor pool in future clinical studies investigating molecular predictors of response to hormonal therapy in LGSOC.

1. Introduction

Low-grade serous ovarian carcinomas (LGSOCs) are an under-characterized histological subtype of epithelial ovarian tumors, accounting for fewer than 5% of all cases of ovarian carcinoma [1]. They are thought to evolve in a stepwise fashion from the ovarian surface epithelium (OSE), ovarian epithelial inclusions, cystadenomas, and borderline

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tumors (SBOTs), with literature data showing that borderline tumors exist on a continuum with low-grade serous ovarian carcinomas, and that the two may share a common pathogenesis. LGSOCs occur at a younger age than high grade serous carcinomas (HGSCs), and are characterized by a longer overall survival despite lower sensitivity to standard chemotherapy [2]. Given the awareness that cytotoxic chemotherapy has limited activity in low-grade serous carcinoma, a search for more effective and/or less toxic systemic therapies is needed. In this context, understanding of molecular events triggering cancer development and of molecular pathways that regulate its biological behavior is imperative to improve treatment outcome by developing curative cancer therapies. Among potential treatment options, hormonal agents have demonstrated some degree of activity in LGSOC [3]. However, few reports are available detailing the expression of hormone receptors in LGSOC tissue specimens (available data mostly referring to ERα and PR) [4,5], investigating their role as a predictive biomarker, or, more in general, exploring the putative signal transduction pathways leading to tumor response after hormonal treatment. Indeed, it is now widely accepted that estrogen signaling is a balance between two opposing forces in the form of two distinct receptors (ERα and ERβ) and their splice variants [6]. ERα and ERβ are members of the nuclear receptor superfamily of ligand-dependent transcription factors and share both structural and functional homologies, although they are encoded by separate genes. They have been shown to induce different biological effects, through the regulation of different genes in response to estradiol (E_2) and selective estrogen receptor modulators, or the modulation of the same genes in opposite directions, in agreement with a *yin/yang* hypothesis. Whereas ERα is proliferative and pro-tumorigenic, ERβ is anti-proliferative, pro-apoptotic and anti-metastatic [6]. Several ERβ isoforms have been reported so far: wild-type ERβ (ERβ1) encodes the full-length, 530 aminoacid receptor protein and is the only fully functional isoform able to bind ligand; ERβ2 to ERβ5, which utilize alternative exons, encode variant receptors with different C-termini and thus do not form homodimers and have no innate activities of their own, but may modulate estrogen action when dimerized with ERβ1 or ERα [6]. Evidence is also accumulating that estrogens exert non-genomic actions through cytoplasmic or cell membrane-bound ER [7]. Remarkably, we recently demonstrated that ERβ isoforms and their sub-cellular localization play a crucial role in HGSOC progression and response to chemotherapy, also providing mechanistic evidence to support clinical data [8–10].

In order to provide insights into the hormone receptor profile and its possible clinical significance in LGSOC, primary and metastatic tissue samples were analyzed for ERα, ERβ1, ERβ2, ERβ5, PR and AR expression. Borderline ovarian tumors (specifically, micropapillary variant of serous borderline tumors) were also included in the study, to acquire knowledge on the potential role of hormones in the pathogenesis of LGSOC.

2. Materials and methods

2.1. Patients

This retrospective study included LGSOC specimens collected for clinical purposes between the years 2001 and 2014 at the Gynecologic Oncology Unit, Catholic University of the Sacred Heart, Rome, Italy. Twenty-five primary tumor samples were analyzed, including matched metastatic lesions for 6 patients. Six patients (median age 48, range 36–74) with micropapillary SBOT were also included in the study: all of them underwent staging procedures with the exception of one 36-yrs. old, nulliparous patient desiring fertility preserving surgery. Stage of disease was: stage IA (N = 2), stage IB (N = 3), and stage IIIA (N = 1, non-invasive implants). Histological classification of ovarian tumors was revised according to the 2014 WHO classification of Tumors of the Female Genital Tract [11]. Typical histopathologic features of LGSOC included mild to moderate nuclear atypia and a low frequency of mitotic figures. In our Institution, a written informed consent is routinely requested from patients for collection of their clinical data, as well as formalin-fixed paraffin embedded sections for research use. Clinical information was obtained from the existing medical records according to institutional guidelines. All data were managed using anonymous numerical codes.

2.2. Immunohistochemical analysis

Immunohistochemical analysis was carried out on three-micrometer-thick paraffin sections as described [9,12]. Conditions for antigen retrieval, incubation times and primary antibodies used are described in Supplementary Table 1. Scoring of hormone receptors was evaluated as previously reported [9]. Briefly, the mean percentage of stained cells was categorized as follows: 0 = negative, 1 = 1–10%, 2 = 11–33%, 3 = 34–66%, 4 = 67–100%. The intensity of staining was also evaluated and graded from 1 to 3, where 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The two values obtained were multiplied to calculate an immunoreactive score (IRS, maximum value 12). Immunohistochemical assessment was carried out by two investigators.

2.3. Immunofluorescence of fixed paraffin-embedded ovarian tissue sections

Three-micrometer-thick paraffin sections were mounted on Superfrost coated slides, and dried overnight. The sections were deparaffinized in xylene, rehydrated in graded solutions of ethanol and rinsed for 5 min in distilled water. Antigen retrieval procedure was performed by microwave oven heating in citrate buffer (pH = 6). Sections were incubated with 20% normal goat serum for 30 min at room temperature (RT) and then incubated at 4 °C overnight with the primary antibody (ERβ2, clone 57/3, Serotec Ltd., dilution 1:100) and COX IV (clone 3E11, Cell Signaling Technology, Manassas, VA, USA, dilution 1:1000, a widely used probe for mitochondria staining). The optimal dilution of the primary antibody had been established before by immuno-enzymatic staining using conventional techniques (two-stage immunoperoxidase technique, DAB). After overnight incubation, slides were washed in TBS and incubated in the dark for 1 h at RT with secondary antibody anti-mouse Alexa Fluor-488 conjugate and anti-rabbit Texas Red conjugate (Thermo Fisher Scientific, Lafayette, Colorado, United States, dilution 1:200). After extensive washing in PBS 1% Tween-20, tissues were stained with DAPI (4′,6-diamidino-2-phenylindole, 1.5 μg/ml) and mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, Ontario, Canada). Slides were observed under the fluorescence microscope (Leica, Milan, Italy) using a 40× or a 100× oil immersion objective.

2.4. Cell culture

VOA-1056 cells were gifts from Dr. Clara Salamanca at Canadian OvCaRe Cell Bank (BC Cancer Agency, Vancouver, Canada) and were cultured in 199/105 medium (Sigma-Aldrich, St. Louis, MO, USA). HOC-7 was a gift from Dr. Kwong-Kwok Wong at MD Anderson Cancer Center, Houston, Texas [13] and were cultured in RPMI 1640 Medium (Lonza, Basel, Switzerland). The immortalized human ovarian surface epithelial cell line (HOSE, T1074) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada) and cultured in Prigrow I Medium (ABM). VOA-1056, HOC-7 and HOSE cell lines were maintained in the specific medium supplemented with 10% FCS, 1% antibiotics, 1% glutamine and 1% MEM (Sigma-Aldrich) in a humidified incubator at 37 °C with 5% CO_2.

2.5. Proliferation assay

VOA-1056 (3.5×10^5 per well), HOC-7 (1.6×10^5 per well), and MCF-7 (1.5×10^5 per well) cells were seeded in 6-well plates in

complete culture medium. After overnight incubation, the medium was changed to phenol-free medium supplemented with 10% CS-FBS (charcoal-stripped serum) and containing various concentrations of 17β-estradiol (E_2, Sigma-Aldrich); 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, a selective ERα agonist, Tocris Bioscience, Ellisville, MO, USA); or 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, a selective ERβ agonist, Tocris Bioscience). Substances were dissolved in absolute ethanol (E_2) or DMSO (PPT, DPN) and diluted in the appropriate culture medium immediately before use. Control cells received the same amount of diluent. The medium was renewed after 48 h. At 120 h of incubation, cells were harvested by trypsinization, and viable cells were counted by Nucleocounter NC-200 (Chemometec A/S, Allerod Denmark) using Via1-Cassettes (Chemometec). Cell proliferation was determined as the percentage change in the total number of cells in comparison with the untreated cells. All experiments were performed at least three times in duplicate. To validate our experimental conditions, the proliferation of MCF-7 cells (ECACC) was assessed following treatment with E_2, DPN or PPT.

2.6. MG132 inhibition

HOC-7 (1×10^4 per well) cells were seeded in 96-well plates in complete culture medium. After overnight incubation, the proteasome inhibitor MG132 (Calbiochem, San Diego, CA, dissolved in DMSO solution buffer at 10 mM as a stock solution) was added at increasing concentrations (0–25 μM). After 24 h of treatment, HOC-7 cell viability was determined with the ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA, USA), according to the manufacturer's protocol. The luminescence intensity was measured with EnSpire multimode plate reader (PerkinElmer). A dose-response curve was generated and the IC_{50} value (50% inhibiting concentration) was determined. The IC_{50} value of 1.56 μM was then used in all subsequent experiments.

2.7. Real time PCR

To evaluate hormone-receptor mRNA levels, RT-qPCR was carried out using analytical procedure and primers previously described in [14]. Relative quantification of target mRNA was performed according to $-\Delta\Delta$Ct method [15]. In each assay, the PCR efficiency was also calculated using serial dilution of one experimental sample; efficiency values between 80 and 100% were found for each primer set and taken into account for the comparative quantitation analysis.

2.8. Western blot analysis

Western blot analysis was performed with a SDS-PAGE Electrophoresis System as described previously [14]. Briefly, equal amounts of protein extract (60 μg/sample) were separated by SDS polyacrylamide gel electrophoresis, blotted to PVDF membrane and sequentially probed with the following primary antibodies: anti-ERα (clone 6F11, Santa Cruz, CA, USA); anti-ERβ total (clone H150, Santa Cruz); anti-PR (clone 16, Leica Microsystems, Bannockburn, IL, USA); anti-AR (clone AR441, Abcam, Cambridge, UK); anti-anti- β-actin (A5441, Sigma-Aldrich) at 4 °C overnight.

2.9. Statistical analysis

Overall survival (OS) was measured from the date of histological diagnosis to the date of death or last seen; disease free survival (DFS) was defined as the interval between primary surgery (histological diagnosis) and date of progression or last seen. Median survival was calculated using the Kaplan-Meier methods. Cell proliferation data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to determine if significant differences existed between groups. Data are reported as mean ± SEM. P values are for two-sided tests. P values ≤0.05 were considered statistically significant. Analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Patients features and outcome

Clinicopathological characteristics of the overall series are summarized in Table 1. The median age of patients was 48 years (range 23–75), within the range of previous studies [16,17]. All patients underwent primary cytoreduction, with complete debulking achieved in all but 3 subjects. At diagnosis, 10 patients were stage I/II and 15 were stage III. Follow-up information was available for all cases with a mean follow-up time of 53 months (9–180) from the date of surgery. Relapse of disease was observed in 6 patients, while deaths occurred in 2 patients; 5-year DFS was 78% (median DFS 73 months), while 5-year OS was 85% (median OS not reached) (Fig. 1). These survival figures well match with the very recent data published by Grabowski et al., [18] and confirm the clinical relevance of upfront surgical debulking in this disease.

3.2. Hormone receptor expression profile in LGSOCs and in matched peritoneal metastases

A total of 25 primary LGSOCs were evaluated. Fig. 2 shows hormone receptor status in the entire series of patients. Medium/high ERα nuclear expression levels were detected in the majority of tumors (80%), whereas only about half of all patients showed medium/high PR expression. Overall in our cohort, ERβ1 and ERβ5 were exclusively expressed at the nuclear level, while ERβ2 also localized in the cytoplasm (Fig. 2A). The majority of patients showed absent or low ERβ1 expression (80%), whereas nuclear levels of ERβ2 and ERβ5 were elevated in all cases. Low cytoplasmic ERβ2 expression levels were detected in 12 out of 25 patients. AR expression was negligible in the entire series. Correlation analysis showed that the six receptors were expressed independently.

After stratification according to menopausal status we did not observe any significant changes in the hormone receptor distribution (data not shown), with the only exception of PR expression showing a significant decrease in post-menopausal compared to pre-menopausal patients (IRS 8.6 ± 1.0 and 3.3 ± 1.2, respectively, mean ± SEM, $p = 0.004$). Likewise, stratification per stage of disease did not show significant differences between early- and advanced-stage patients (data not shown).

In order to better clarify the role of hormones in LGSOC development we immunostained matched metastases from 6 patients. Interestingly

Table 1
Clinicopathological features of the overall series.

Characteristics	No. of patients (%)
All cases	25
Median Age, years (range)	48 (23–75)
Menopausal status	
Pre-menopause (median age 40)	15 (60)
Post-menopause (median age 68)	10 (40)
Type of primary surgery	
Cytoreduction	25
Residual tumor after primary surgery (cm)	
0	22 (88)
<1	3 (12)
FIGO Stage	
I	9 (36)
II	1 (4)
III	15 (60)
Primary chemotherapy	
None	3 (12)
Platinum/paclitaxel	17 (68)
Platinum-based	4 (16)
Unknown	1 (4)

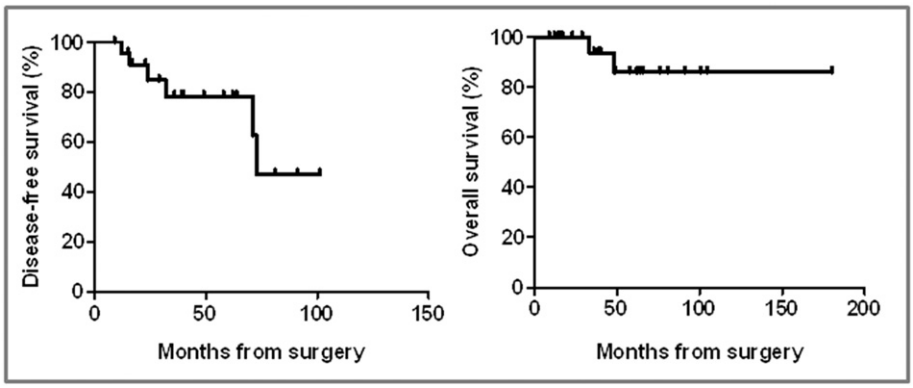

Fig. 1. Kaplan–Meier curves of disease-free survival (A) and overall survival (B) for the entire series of patients.

we found that cytoplasmic ERβ2 expression was considerably increased in the metastatic lesion when compared to primary tumor (Table 2, Fig. 2B). No other relevant differences between primary tumors and the matching metastases were observed in this limited series of cases.

3.3. Hormone receptors in borderline ovarian cancers

In order to shed light on the potential role of hormones in the malignant progression of the precancerous lesion to cancer, we assessed the hormone receptor profile status in micropapillary SBOTs. As shown in Fig. 3, micropapillary SBOTs and LGSOCs showed an almost comparable profile, except for ERβ1 protein levels which displayed a considerable higher expression in micropapillary SBOT compared to LGSOC (IRS score 10.3 ± 1.1 and 2.2 ± 0.5, respectively). A similar trend, although less marked, was also observed for AR immunostaining (3.7 ± 0.8 and 1.2 ± 0.2, respectively).

3.4. Expression of steroid hormone receptors in LGSOC cell lines and regulation of cell growth by selective ligands

To investigate a putative role for estrogens in LGSOC, we first determined the expression of hormone receptors by RT-qPCR and WB analyses in two primary human LGSOC cell lines, i.e. HOC-7 and VOA-1056, and in human immortalized ovarian epithelial cells (HOSE) for comparison. MCF-7 cells were used as positive control. RT-qPCR analysis showed ERα mRNA expression at a very low level only in HOC-7, whereas VOA-1056 and HOSE cells were negative. On the other hand, all cell lines were shown to express the three ERβ transcript variants tested (i.e. ERβ1, ERβ2 and ERβ5). Progesterone receptor and AR transcripts were detected at low levels in HOSE and in VOA-1056 cells only. There was a concordance between gene and protein expression for ERα and for the three ERβ isoforms, these latter identified with the ERβ H150 antibody, as previously reported [14,19]. Conversely, PR and AR proteins were undetectable in all cell lines (Fig. 4A).

In order to examine if the activated estrogen pathway is essential for the proliferation of LGSOC cells, proliferation of HOC-7 and VOA-1056 was evaluated 120 h following treatment with E_2 or selective ERs agonists (i.e. PPT or DPN). In our experimental conditions, cell growth was not affected by either the endogenous or the selective synthetic ligands (Fig. 4B). MCF-7 cells used as positive control gave results (data not shown) consistent with literature data [14,20]. Overall, these data indicate that E_2 treatment of ERα-negative cell lines has no effects on LGSOC cell proliferation.

3.5. Regulation of ERs levels in LGSOC cell lines

It has been reported that up-regulation of proteasome subunit levels occurs in LGSOC *in vitro* and *in vivo* [21], and that the ubiquitin-proteasome pathway is an important regulator of ER cellular levels [22,23]. These evidences prompted us to evaluate whether ERs expression in LGSOC cell lines might be changed following treatment with the proteasome inhibitor MG132. Interestingly, we found that MG132 treatment *per se* markedly increased the levels of ERβ1 while not changing ERβ2 and ERβ5 isoform, as well as ERα expression (Fig. 4C) in HOC-7 cells, proving the proteasome-mediated degradation of the ERβ1 protein in this cell line. As expected, a 24 h exposure of HOC-7 cells to MG132 considerably decreased cell viability, with an IC50 value of 1.56 ± 0.5 μM.

4. Discussion

Studies have shown that low-grade serous carcinoma is less sensitive to conventional chemotherapy in the neoadjuvant, adjuvant, or recurrent settings than high-grade ovarian cancer [2,17]. Despite the low response-rate, the platinum-taxane doublet remains the standard of care in clinical practice, at least in the adjuvant setting, while in the recurrent setting, the currently available therapeutic options include conventional chemotherapy or hormonal therapy [24]. The use of hormonal therapy as a component in the treatment of relapsed disease is mainly based on the results from the MD Anderson group, showing complete or partial response to hormonal therapy in a small fraction of LGSOC (9%) and disease control in 62% of patients [3]. Importantly, the same group of investigators recently demonstrated, in a retrospective cohort, that women with stage II-IV LGSOC who received hormonal maintenance therapy following primary treatment had statistically significant improvement in PFS compared with women who underwent surveillance [25]. These promising results suggest that hormonal manipulation might be effective for controlling the growth of LGSOC, although many questions still remain [26].

Among these, the role of the ERα/PR status and of the co-expression of ERβ isoforms in predicting response to hormonal therapy needs to be further explored. Such a detailed characterization is indeed important since there is evidence showing that the relative hormone receptor expression in target cells may determine diverse cell responses [6]. In this context, here we show for the first time that along with ERα and PR, LGSOC also express the ERβ isoforms, namely ERβ1, ERβ2 and ERβ5. Overall, cancer tissues were shown to express high levels of nuclear ERα, ERβ2 and ERβ5, while the wt ERβ protein (i.e. ERβ1) was present only at very low levels. These findings may be particularly relevant when considering that the relative levels of ERα and ERβ isoforms in the total receptor pool may be more important predictors of disease and/or of response to therapy than their absolute values. Indeed, at the promoters of some genes, particularly those involved in proliferation, ERα and ERβ can have opposite actions, a finding which suggests that the overall proliferative response to E_2 is the result of a balance between ERα and ERβ signaling [27]. This scenario is further complicated by the presence of different ERβ isoforms that have been shown to present different localization patterns in living cells, and to exhibit different properties [6,27]. Interestingly, the comparison of primary tumor with

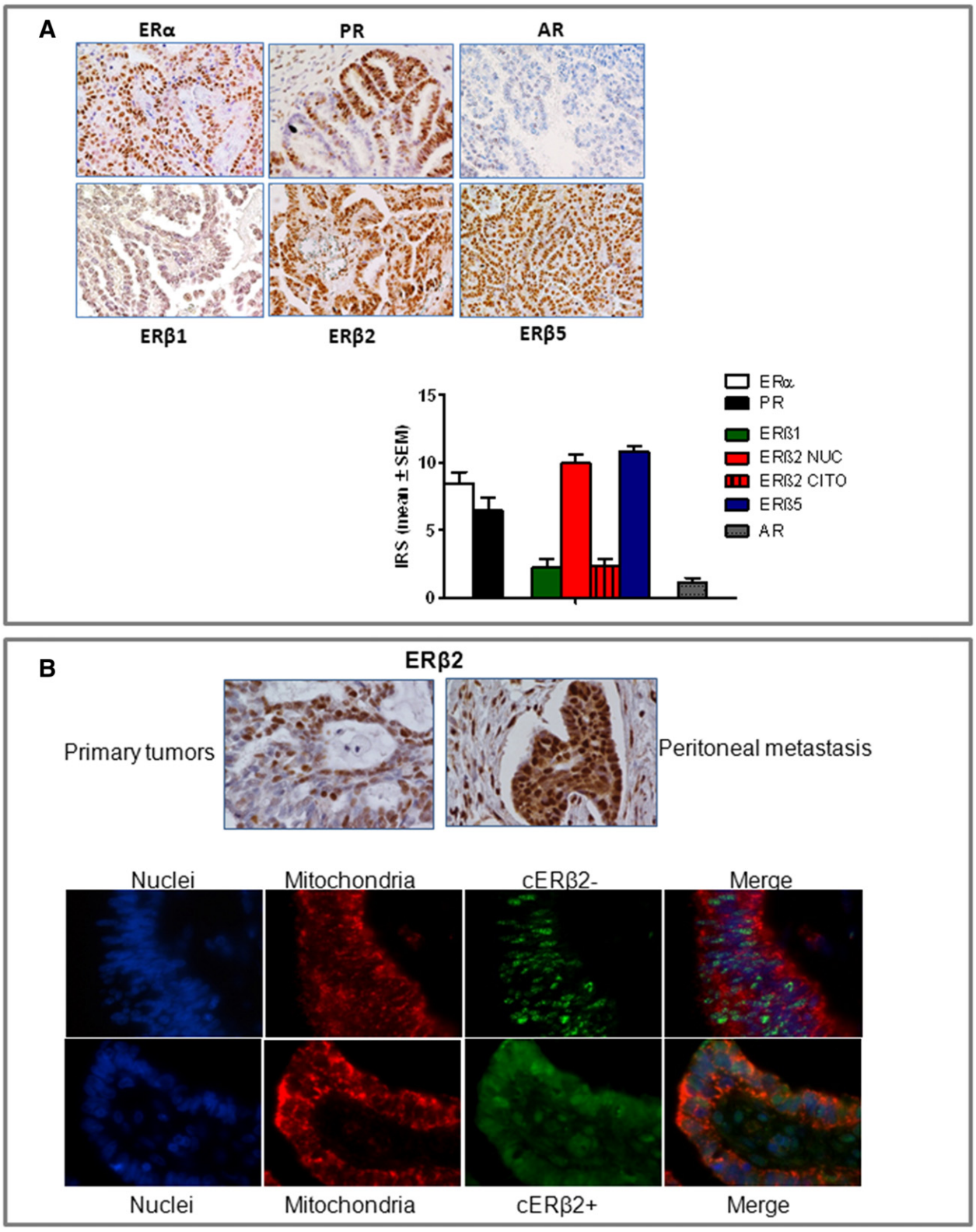

Fig. 2. A) Representative pictures for ERα, ERβ1, ERβ2, ERβ5, PR, and AR immunostaining in LGSOC patients (magnification 20×) and bar chart showing levels of hormone receptor expression (mean ± SEM, n = 25). Results are expressed as IRS (Immunoreactive receptor score). B) Representative pictures showing exclusive nuclear and nuclear/cytoplasmic ERβ2 expression in primary tumor and matched peritoneal metastasis. Fluorescence microscopy was also used to confirm localization of ERβ2 in primary tumor and matched peritoneal metastasis. Nuclei were stained with DAPI (blue), mitochondria with COX IV (red) and ERβ2 (green). Merged images in B shows the nuclear (upper row) and mitochondrial (lower row) localization of cytoplasmic ERβ2.

the matched metastasis (available only for 6 patients) revealed a comparable hormone receptor profile, with the exception of the ERβ2 isoform, this latter showing a significant increase in cytoplasmic expression in the metastatic lesions compared to the paired primary tumors. In this context, it has to be acknowledged that ERβ2 is a mitochondrial component in cancer cells and it has been shown to exert an

Table 2
Hormone receptor profile in primary tumors and paired metastases.

Case	Tissue	Age	ERα	PR	ERβ1	ERβ2 Nuc	ERβ2 Cyt	ERβ5	AR
# 1	Primary	28	12	3	2	6	1	8	3
	Metastasis		12	6	12	12	6	12	2
# 2	Primary	34	9	3	0	12	4	12	0
	Metastasis		9	3	4	6	6	6	0
# 3	Primary	35	9	12	1	9	4	12	1
	Metastasis		12	3	4	12	0	6	2
# 4	Primary	39	3	3	8	12	4	12	0
	Metastasis		4	6	2	6	6	6	0
# 5	Primary	44	4	12	3	12	0	12	0
	Metastasis		9	6	4	12	6	12	4
# 6	Primary	73	12	0	12	12	0	12	1
	Metastasis		6	0	12	12	12	12	0

Hormone receptor expression is reported as immunoreactive score (IRS, see Material and methods for calculation).

anti-apoptotic role in advanced HGSOC: in particular, we demonstrated that mitochondrial ERβ2, interacting with BAD, precludes the Bcl-xL/BAD heterodimer formation, a condition that, in turn, inhibits Bax oligomerization, release of cytochrome c, and ultimately apoptosis [10]. Since apoptotic escape has been shown to play an important role in facilitating metastatic process [28], it is conceivable that the higher cytoplasmic ERβ2 levels in metastases compared to matched primary tumor could sustain an aggressive tumor phenotype also in LGSOC.

LGSOCs are often associated with typical SBOT; these tumors are considered nonaggressive, although the rate of potential progression to LGSOC, which is around 5–10% in the whole series, rises in cases with peritoneal implants [29,30]. Micropapillary subtype has a more aggressive clinical behavior, and it has been reported to have a higher risk of progression, possibly representing an intermediate step in development to LGSOC [30–32]. Therefore, we decided to focus the attention on the analysis of steroid hormone receptor profile in this specific variant of SBOT in comparison with LGSOC. Interestingly, ERβ1 was the only molecular biomarker showing a different expression between micropapillary SBOT and LGSOC, with markedly higher levels in the precancerous lesions. This finding is in line with previous data suggesting that a decreased expression of ERβ in cancer tissue compared to normal ovary represents a feature of malignant transformation [6,8,33,34]. In the light of mechanistic studies supporting an anti-proliferative and pro-apoptotic role of ERβ1, we could speculate that higher ERβ1 expression in micropapillary SBOT might indeed constitute a self-protective mechanism against tumor proliferation, while its expression is lost in early stages of LGSOC.

These data prompted us to investigate in *in vitro* models, the molecular mechanisms through which estrogens can modulate the growth of LGSOC. However, the molecular characterization of the hormone receptor profile of HOC-7 and VOA-1056 cells, demonstrated the lack or very low levels of ERα, PR and AR in both cell lines, while ERβ isoforms were all present (although at different levels), on the whole a phenotype not

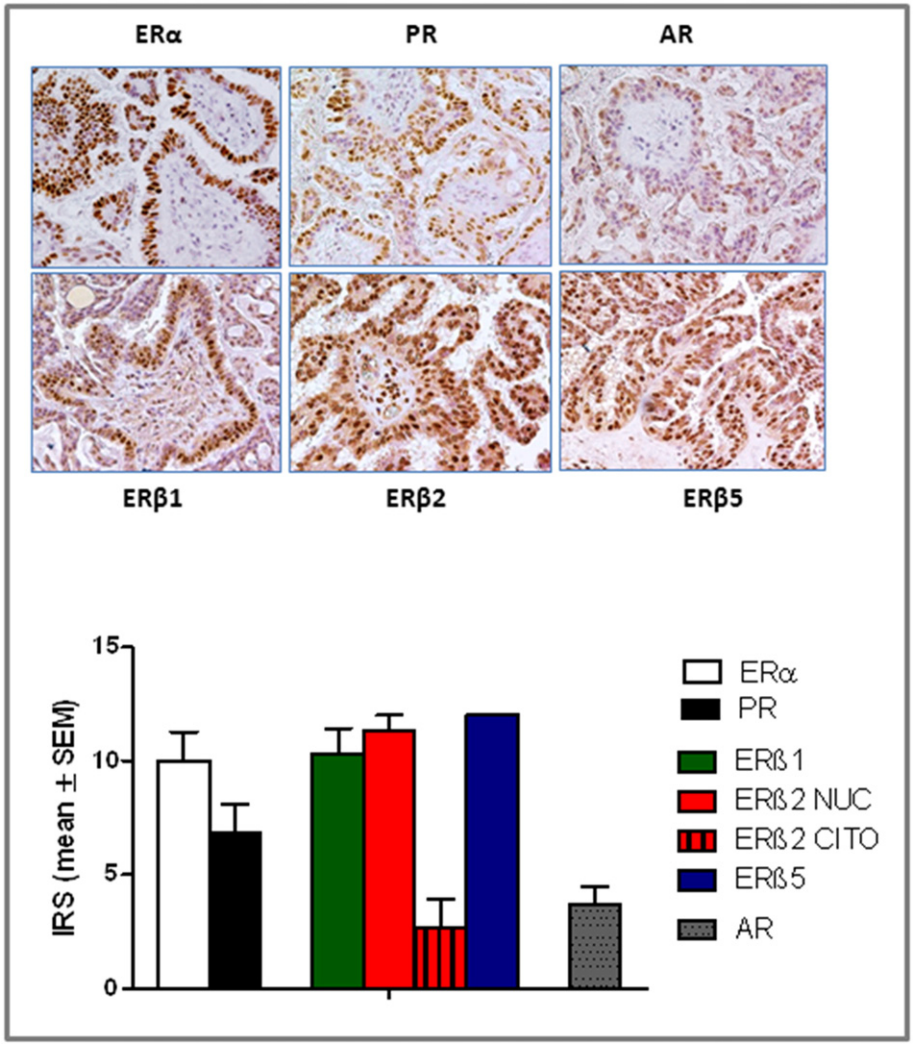

Fig. 3. A) Representative pictures for ERα, ERβ1, ERβ2, ERβ5, PR, and AR immunostaining in patients with borderline ovarian tumors (magnification 20×) and bar chart showing levels of hormone receptor expression (mean ± SEM, n = 6). Results are expressed as IRS (Immunoreactive receptor score).

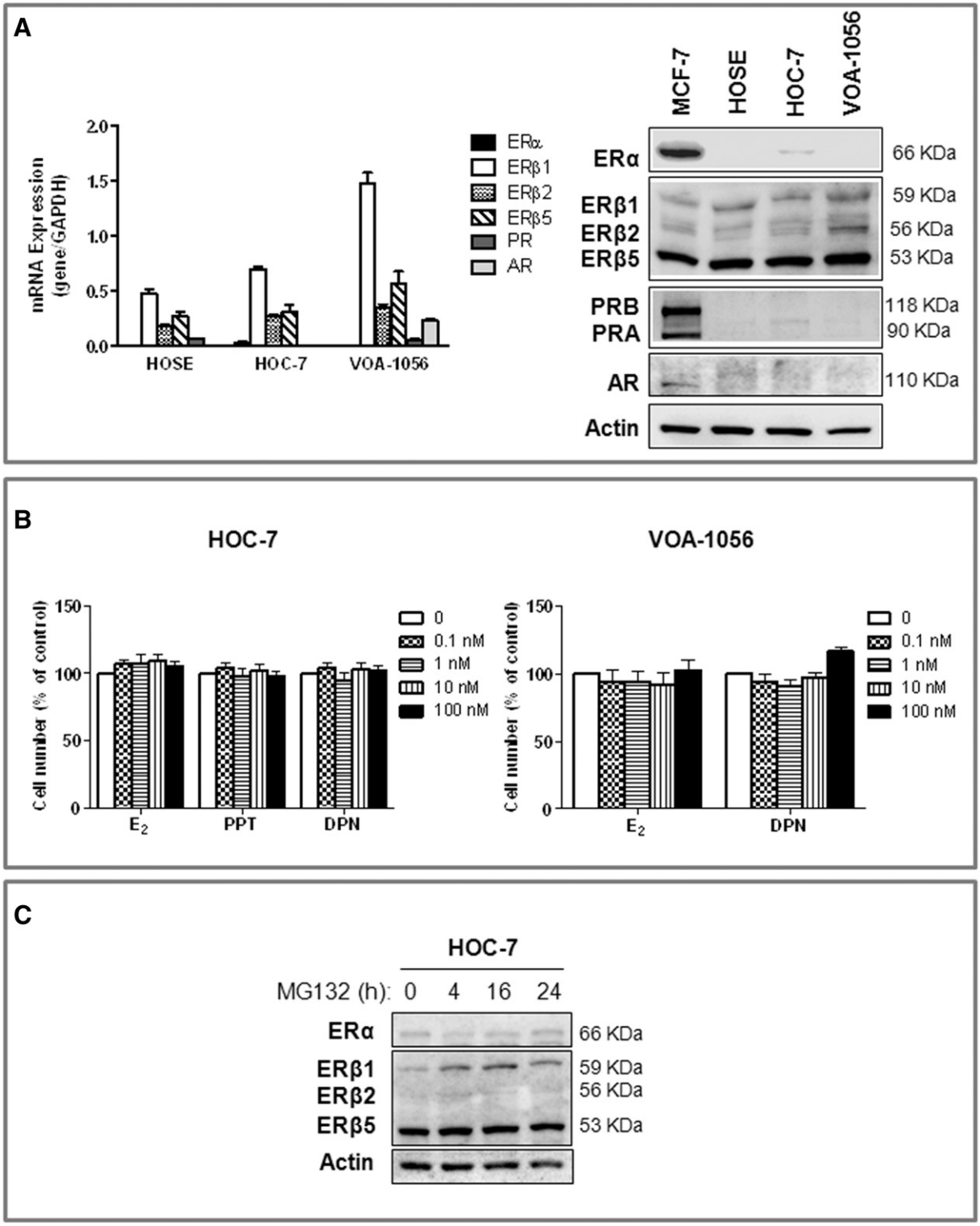

Fig. 4. A) Expression analysis of steroid hormone receptors in low-grade serous ovarian cancer (LGSOC) cell lines HOC-7 and VOA-1056, and in HOSE (immortalized human ovarian surface epithelial cell line). Cells were grown in complete culture medium and MCF-7 cells were used as positive control. The relative mRNA expression of hormone receptors was evaluated by RT-qPCR, utilizing specific set of primers. All samples were normalized to the housekeeping gene, GAPDH. The results are presented as fold change for each mRNA in LGSOC cell lines and HOSE compared to MCF-7 cells. Results are expressed as the mean ± SEM derived from at least three different experiments. Representative Western blot of hormone receptors expressions. Protein levels were determined by subjecting 60 μg of protein extract to SDS-gel electrophoresis, followed by Western blotting using specific antibodies. β-actin was used as a control of equal sample loading. B) Effects of E_2, the ERα-selective agonist PPT, and the ERβ-selective agonist DPN, on LGSOC cell growth. Cells were treated with various concentrations of substances in phenol-red free medium supplemented with charcoal stripped FBS. Concentrations are expressed in nanomolar. Control cells received the same amount of diluent. The medium was renewed after 48 h. At 120 h of incubation viable cells were counted using Nucleocounter. All results are expressed as the mean ± SEM derived from at least three different experiments. C) Representative Western blot of hormone receptors expressions, at different time points following treatment of HOC-7 cells with IC_{50} MG132, a proteasome inhibitor. β-actin was used as a control of equal sample loading.

entirely resembling the common clinical situation. Proliferation studies proved that the growth of LGSOC cell lines was not affected by either the endogenous or the selective synthetic ligands, in line with reports indicating that ERα expression is necessary and sufficient to induce the E_2-stimulated growth of epithelial ovarian cancer cells in *in vitro* models [6,35]. However, we cannot completely rule out other possible causes of the observed lack of modulation, including the actual presence and the effective function in our experimental systems of important auxiliary factors (i.e. co-activator or co-inhibitor protein) that are necessary for transcriptional activation of target genes by hormone receptors, and that are known to be cell- and tissue-specific [36]. Further studies are needed to fully uncover the role of ERα/ERβ-mediated estrogen signaling in LGSOC, provided that suitable experimental models are selected. Also, given the potential importance of ERs in ovarian cancer and the possibility to use selective targeting of hormone receptor subtypes for therapy, research should define which and how cellular systems control ERs expression, since this could be a critical step in cancer characterization.

In this context, it is worthy to note that some lines of evidence suggest that the up-regulation of proteasome protein levels is a phenomenon occurring in different ovarian cancer histotype, including LGSOC [21,37,38]. The role of ubiquitin-proteasome pathway as an important regulator of ERs cellular levels is known, as well [22,23]. These findings led us to hypothesize that the very low levels of ERβ1 occurring in patient tissue samples might be the consequences of a high proteasomic activity. Using the HOC-7 cell line as a model we indeed found that treatment of LGSOC cells with MG132, a known proteasome inhibitor, caused the accumulation of the ERβ1 isoform, while not changing significantly the expression of the other two isoforms nor ERα. The increase in ERβ1 cellular levels after MG132, along with the decreased cellular proliferation, may suggest potential therapeutic implication for targeting this pathway. However, other mechanisms might also account for the decreased ERβ1 expression, since down-regulation of the ERβ gene has been attributed to methylation in a variety of tissues including ovarian cancer [39]. Specifically, owing to promoter hypermethylation, the mRNA levels of ERβ1, ERβ2 and ERβ4 were shown to be significantly lower in ovarian cancer tissues than in their corresponding normal counterparts [33].

In conclusion findings from the present study, that have to be confirmed in larger trials, highlight the need of assessing the hormonal receptor status in future clinical studies investigating molecular predictors of endocrine therapy responsiveness in LGSOC. Besides, further research is warranted to investigate the contribution of ERα and ERβ isoforms in determining the pathogenic role of estrogen signaling in LGSOC development and progression, also in the light of new active research on compounds selectively targeting ERβ [40].

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Conflict of interest statement

We have no conflicts of interest.

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