# hLH/hCG-receptor expression correlates with *in vitro* invasiveness in human primary endometrial cancer

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## Abstract

*Objective.* Endometrial cancer (EC) is the most frequent cancer of the female genital tract. It has been hypothesized that those ECs that occur in the postmenopausal period, might be sensitive to elevated levels of luteinizing hormone/human chorionic gonadotropin (LH/hCG). Based on previous indications, we analyzed the functional expression of LH/hCG receptors (LH/hCG-R) in primary ECs.

*Methods.* We studied a cohort of primary ECs, in which both the *LH/hCG-R* mRNA and the LH/hCG-R protein were analyzed. Results were correlated with both clinical–pathological data and the effects of LH addition on cell invasion *in vitro*.

*Results.* The *LH/hCG-R* mRNA levels ranged from $4.67 \ e^{-02}$ to $2.36 \ e^{+03}$. The transcript was properly translated into a functional LH/hCG-R protein. The analysis of cell invasion *in vitro* in response to LH/hCG allowed us to divide the EC samples into two groups, one with a null or very low response (non-responders=NR) and the other with a significant response to LH (responders=R). The two groups had significantly different levels of *LH/hCG-R* mRNA expression: the NR group had a median value of $1.40 \ e^{+00}$, while the R group of $7.42 \ e^{+01}$ ($p=0.043$).

*Conclusion.* In primary ECs a statistically significant correlation emerged between the levels of *LH/hCG-R* mRNA and the LH-induced cell invasion *in vitro*. These results suggest that therapies aimed at decreasing LH levels, through Gonadotropin Releasing Hormone (Gn-RH) analogues, could produce benefits in the treatment of recurrent or metastatic EC, especially in patients displaying high LH/hCG-R levels.
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*Keywords:* hLH/hCG-receptor; Invasiveness; Endometrial cancer

## Introduction

Endometrial cancer (EC) is currently the most frequent malignancy of the female reproductive tract [1]. EC is a curable malignancy, with a good prognosis and an overall survival rate of about 80%. This is mainly due to the fact that the majority of cases are diagnosed in the early stages (International Federation of Gynecology and Obstetrics (FIGO) Stage I). However, there is a group of patients with a high risk of cancer recurrence or metastatic spread. In particular, those cancers arising in women in an advanced menopausal phase are often more aggressive and apparently independent from estrogen (E) secretion [2]. The latter, especially when unlinked to progesterone, is apparently one of the most relevant etiologic factors in EC [3], since Es contribute to the malignant transformation through a potent mitogenic effect on the endometrial surface epithelium [4]. It was hypothesized that E-independent ECs might be sensitive to the elevated levels of luteinizing hormone/human chorionic gonadotropin (LH/hCG) that characterize the post-menopause. Hence, gonadotropins might be involved in the natural history of at least some types of ECs. This hypothesis was indirectly supported by the demonstration that the LH/

hCG-receptor (*LH/hCG-R*) gene was expressed in human ECs [3] and that the addition of LH/hCG regulates proliferation in EC cell lines [5]. On these bases some clinical trials were performed with the aim of treating patients affected by EC with gonadotropin releasing hormone (Gn-RH) analogues, in order to decrease LH levels. Conflicting results emerged from these studies [5–11].

We performed a study that showed the functional expression of LH/hCG-R in the moderately differentiated EC cell line Hec1A [12]. At a difference from what was reported for Ishigawa cells [13], the addition of LH/hCG to Hec1A cells induces an increase of cell invasion through Matrigel, which is mediated by the activation of protein kinase A. We also provided preliminary evidence that the *LH/hCG-R* mRNA is expressed in primary EC and that primary EC cells can be triggered to invade Matrigel by LH addition, through a PKA-dependent mechanism [12].

To confirm and deepen these results, we studied a larger cohort of primary ECs, in which the quantitative expression of *LH/hCG-R* mRNA and the presence of the LH/hCG-R protein were analyzed. Results were correlated with clinical–pathological data as well as with the effects of LH addition on cell invasion *in vitro*.

## Materials and methods

### Chemicals

MATRIGEL® Basement Membrane Matrix by Becton Dickinson Labware (Becton Dickinson) was used at a final concentration of 250 μg/ml following the instructions. Anti-LH-R (Santa Cruz Biotechnology) (WB: 1:300); anti-αTubulin (WB: 1:500), anti-rabbit peroxidase-conjugate (Sigma) (1:10000), anti-mouse peroxidase-conjugate (1:5000) antibodies are all from Sigma. hrLH Luveris® (Lutropina alfa) 75 UI (3.4 μg) (Serono Laboratoires) was used at 0.3 UI/ml [12]. The monoclonal antibody anti LH-R 3B5 was provided by Dr. Wimalasena and used at a 1:50 final dilution.

### Clinical data

Tissue samples were obtained after informed written consent from 25 consecutive patients who underwent radical surgery with curative intent for primary EC at the Department of Gynecology, Perinatology and Human Reproduction, University of Florence. Uterus was removed and sampling of neoplastic tissue was immediately performed.

### Matrigel invasion assay

Cell suspension from primary samples performed as in [14], were resuspended in DMEM containing 250 μg/ml of heat inactivated BSA (DMEM+BSA). The Matrigel invasion assay was performed as previously described [12]. Due to the different sizes of tissue biopsies, variable concentrations of cells were used (from 50 to $400 \times 10^3$ cells/ml). Four pivotal experiments were performed to ensure that different concentrations in the cell suspension did not interfere with experimental results.

### Immunohistochemistry (IHC)

IHC was performed on 7 μm sections adhered on positive-charged microscope slides. The 3B5 monoclonal antibody was diluted in UltraVBlock (LabVision): PBS 1:10 (v/v). Detection was carried out using a commercially available kit (PicTure Plus Kit, Zymed) as well as DAB chromogen solution (Zymed), according to the manufacturer's instructions.

### RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was promptly extracted from cell suspensions obtained from fresh tissue sample as in [12].

### Real-Time Quantitative PCR (RQ-PCR)

*LH/hCG-R* mRNA was quantified by RQ-PCR, with the ABI PRISM 7700 Sequence Detection System (Applied Biosystem). The β-*glucuronidase* (*GUS*) gene was used as standard reference. The relative expression of *LH/hCG-R* was calculated by using a comparative threshold cycle method. Standard curves were prepared using 10 fold serial dilutions of cDNA retrotranscribed from testis, which represent a positive control for *LH/hCG-R*. The primer sequences were as follows: *LH/hCG-R*: sense GCAGAA-GATGCACAATGGAG; antisense CTCTCAGCAAGCATGGA-AGA; *GUS* sense GAAAATATGTGGTTGGAGAGCTCATT; antisense CGGAGTGAAGATCCCCTTTTTA. The primers were used at a final concentration of 300 nM for both *GUS* and *LH/hCG-R*. Each PCR program started with an incubation at 95 °C for 10 min, followed by 40 cycles of amplification, each involving an annealing–extension step at 60 °C for 1 min and denaturation at 95 °C for 15 s. To exclude contamination of unspecific PCR products, melting curve analysis was always applied to final PCR products after the cycling protocol.

### Protein extraction and Western blot

Protein extraction and Western blots were performed as reported in Crociani et al. [15], loading 60 μg of protein extracts. The immunoreactivity was determined by an enhanced chemiluminescent reaction (Super Signal; Pierce).

### Statistical methods

Statistical analysis was carried out using Stata software (9.1 version, Statacorp; College Station). The Anova test and the *T*-test were used to assess whether the means of two independent groups were *statistically* different from each other. The relationships between LH/hCG-R mRNA expression and IHC data and clinical features (post-operative histology, grading and myometrial infiltration) were evaluated through the Fisher's exact test. $P$ values less than 0.05 were considered statistically significant.

## Results

We collected twenty five consecutive samples from patients who underwent radical surgery with curative intent for primary EC. The clinical–pathological characteristics of the patients are reported in Table 1.

We first determined the expression of *LH/hCG-R* mRNA by nested PCR in all the samples examined (not shown). Subsequently, a quantitative estimate of *LH/hCG-R* mRNA was performed by Real-Time Quantitative PCR (RQ-PCR). In Fig. 1A are reported the typical RQ-PCR amplification curves of *LH/hCG-R* (top) and of the control gene *GUS* (bottom) on representative endometrial samples. A signal of *LH/hCG-R* from EC can be observed starting from cycle 17, evidencing that *LH/hCG-R* is indeed expressed in EC. Panel 1B illustrates dissociation curves from a representative run: the dissociation curve plot displays a characteristic melting peak typical of a single target nucleic acid sequence, confirming the specificity of the reaction. The levels of the *LH/hCG-R* transcript calculated as reported in Materials and methods, were normalized on the levels of the corresponding transcript detected in Hec1A cells. Data obtained through this method showed that the range of *LH/hCG-R* mRNA expression in EC strongly varied among the different samples. In fact levels of the transcript ranged from $4.67\ e^{-02}$ to $2.36\ e^{+03}$, with a median value of $4.00\ e^{+01}$ (see Fig. 1C).

We then confirmed the proper translation of the *LH/hCG-R* mRNA through a WB analysis performed in some of the samples collected (Fig. 2A). The blots showed the presence of two protein bands weighting 85 and 75 kDa, respectively, both attributable to the LH/hCG-R. These two bands represent the mature and the immature forms of the receptor, respectively [16–19]. Hence, the LH/hCG-R protein is expressed both in normal and neoplastic endometrium. The quantitative analysis, comparing the LH/hCG-R bands with those of tubulin, indicates that the LH/hCG-R is expressed at higher levels in adenocarcinoma samples as compared to normal mucosa, confirming what was previously reported by Rao et al. [20].

An IHC analysis was also performed on paraffin embedded samples of the tumours collected. In Fig. 2B representative immunostaining patterns of LH-R expression in four EC specimens are reported. In any case, the positive staining is shown as a brown pigment, with a blue nuclear hematoxylin counterstain. A human testis specimen, which shows a strong intensity of LH-R labelling, is reported in the inset to panel 1, as positive reference. Samples in panels 1 and 2 display a good and diffuse staining, mainly localized in the cytoplasm and plasma membrane of epithelial cells (see arrows), although a faint signal can be also detected in stromal cells. The sample in panel 3 shows a moderate staining, largely confined to the glandular epithelium (see arrow), whereas a sample negative for LH/hCG-R is reported in panel 4. All the results of the IHC analysis are summarized in Table 1 and reported as − (no immunostaining) or + (positive immunostaining). The IHC analysis showed that 65% (15/23) of EC samples expressed the LH/hCG-R. No statistically significant correlation emerged

Table 1
Patients' characteristics, LH-R expression and effect of LH on cell invasion

| Patient | Age | Post-operative histology | Grading | LH/hCG-R expression (IHC) | Cell invasion (untreated samples) (mean±SEM) | Cell invasion (LH-treated EC samples) (mean±SEM) | Effect of LH on cell invasion (% of control) |
|---|---|---|---|---|---|---|---|
| 1 | 68 | EA | G1 | + | 442.5±43.5 | 463.3±133.83 | 105 |
| 2 | 64 | EA | G1 | − | 55±18 | 93.3±16 | 170 |
| 3 | 78 | EA | G3 | + | 90.3±17 | 154±5 | 170 |
| 4 | 64 | EA | G2 | + | 71.7±13.2 | 74±16.56 | 103 |
| 5 | 59 | EA | G2 | + | 28.7±7.3 | 74±26 | 258 |
| 6 | 65 | PSC | G3 | − | 233±33 | 304±44.3 | 130 |
| 7 | 56 | EA | G2 | + | 18±1 | 27±16 | 150 |
| 8 | 63 | EA | G2 | + | 229±48.2 | 252.5±91.5 | 110 |
| 9 | 73 | EA | G3 | − | 25.8±3 | 38±12 | 147 |
| 10 | 64 | EA | G2 | + | 130±21 | 212±36 | 163 |
| 11 | 80 | EA | G1 | + | 42.5±12 | 53.3±9.6 | 125 |
| 12 | 77 | EA | G2 | . | 76.3±17.8 | 195.3±21.7 | 256 |
| 13 | 67 | EA | G2 | − | 142.7±24.8 | 319.7±59.8 | 224 |
| 14 | 71 | EA | nd | − | 10.3±1.7 | 14.5±4 | 140 |
| 15 | 67 | EA | G1 | − | 63.3±9.7 | 63.4±5.7 | 100 |
| 16 | 49 | EA | G3 | + | 237.3±53.3 | 238±54.6 | 100 |
| 17 | 56 | EA | G3 | + | 263±24 | 316±97 | 120 |
| 18 | 72 | EA | G2 | + | 115±18 | 216±68 | 188 |
| 19 | 70 | PSC | G3 | . | 10.9±0.8 | 9.5±0.8 | 88 |
| 20 | 56 | EA | G2 | − | 9±3.7 | 16.2±5.1 | 180 |
| 21 | 63 | EA | G3 | − | 50±6.8 | 45±11 | 90 |
| 22 | 62 | EA | nd | − | 22±2 | 19.7±4 | 90 |
| 23 | 67 | EA | G1 | + | 23±11 | 54±14 | 235 |
| 24 | 71 | EA | G3 | + | 12±2.5 | 13.2±2.2 | 110 |
| 25 | 55 | EA | G2 | − | 112.7±3.7 | 135±26.5 | 120 |

Clinical–pathological characteristics of the patients enrolled in the study are reported. LH/hCG-R expression (IHC) is shown as − (no immunostaining) or + (positive immunostaining). Cell invasion assay results are shown as the mean±SEM before and after LH addition and as folds of increase.
EA: endometrioid adenocarcinoma; PSC: Papillary Serous Carcinoma; nd=not determined.

**A**



**B**

**C**

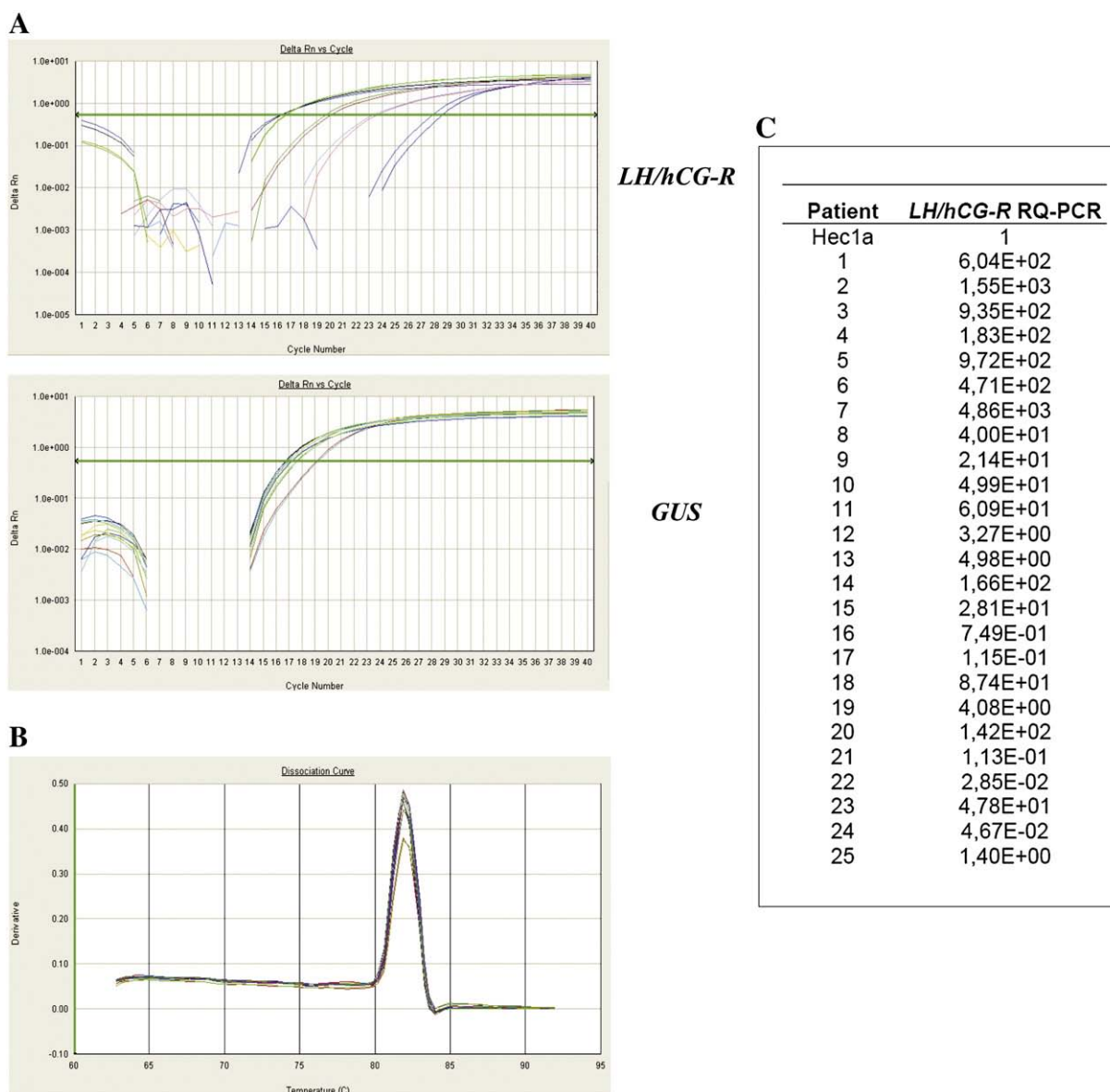| Patient | *LH/hCG-R* RQ-PCR |
|---------|-------------------|
| Hec1a | 1 |
| 1 | 6,04E+02 |
| 2 | 1,55E+03 |
| 3 | 9,35E+02 |
| 4 | 1,83E+02 |
| 5 | 9,72E+02 |
| 6 | 4,71E+02 |
| 7 | 4,86E+03 |
| 8 | 4,00E+01 |
| 9 | 2,14E+01 |
| 10 | 4,99E+01 |
| 11 | 6,09E+01 |
| 12 | 3,27E+00 |
| 13 | 4,98E+00 |
| 14 | 1,66E+02 |
| 15 | 2,81E+01 |
| 16 | 7,49E-01 |
| 17 | 1,15E-01 |
| 18 | 8,74E+01 |
| 19 | 4,08E+00 |
| 20 | 1,42E+02 |
| 21 | 1,13E-01 |
| 22 | 2,85E-02 |
| 23 | 4,78E+01 |
| 24 | 4,67E-02 |
| 25 | 1,40E+00 |

Fig. 1. *LH/hCG-R* expression in primary EC samples. RNA was extracted from cell suspension obtained from fresh EC samples. RQ-PCR was performed using the Sybr Green method. (A) *LH/hCG-R* (upper panel) and *GUS* (lower panel) real-time PCR application curves. Each curve was generated in triplicate from the same sample. (B) Dissociation curves of the samples analyzed by RQ-PCR. The plot of the first derivative of the rate of change in fluorescence as a function of temperature is shown. (C) *LH/hCG-R* mRNA expression levels in all the patients enrolled in the study.

between IHC and RQ-PCR data ($p=0.58$), nor of RQ-PCR or IHC data with the clinical–pathological characteristics of the patients (post-operative histology, grading and myometrial infiltration) (Fig. 3A).

Based on previous data [12], we evaluated the effect of LH addition on cell invasion through Matrigel in all the primary samples collected. Results are shown in Table 1, where both the values before and after LH addition (mean±SEM) and the percentage increase of cell invasion after LH addition are reported. The response to LH addition varied among the different samples: some were almost unaffected by LH addition, while others displayed a 2/2.5 fold increase in cell invasion after LH addition. Using a cut-off close to the median value of the percentage increase of cell invasion after LH addition we divided the entire EC population into two groups, one with a null or

very low response to LH ($\leq 1.2$ fold) (non-responders$=$NR) and the other with a significant response to LH addition ($>1.2$ fold) (responders$=$R). The two groups had significantly different levels of *LH/hCG-R* mRNA expression (Fig. 3B). In fact, the median value of the NR group was 1.40 $e^{+00}$, while that of the R group was 7.42 $e^{+01}$ ($p=0.043$).

**Discussion**

In the present paper we measured LH/hCG-R expression at both the mRNA and protein levels in a cohort of primary ECs. LH/hCG-R expression was analyzed in conjunction with clinical–pathological features of the samples collected, as well as with the effects of LH addition on cell invasion *in vitro*. The main result that emerged from our study was a statistically
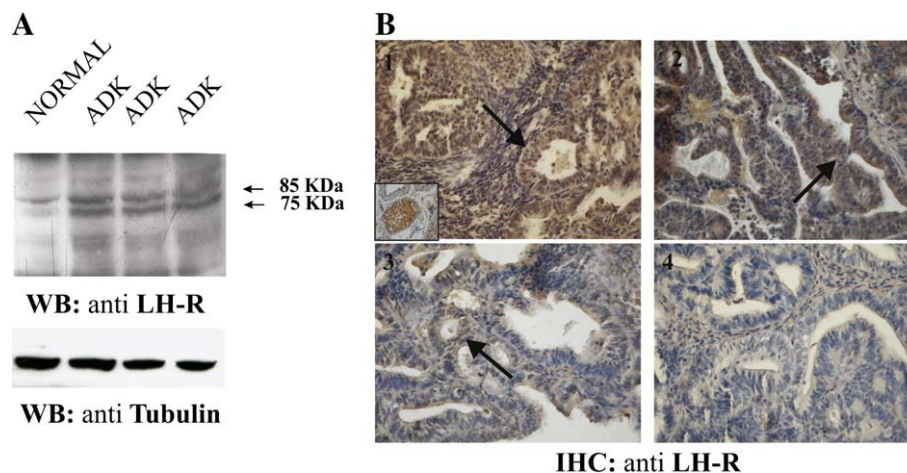
Fig. 2. Expression of LH/hCG-R. (A) LH/hCG-R protein expression in normal and neoplastic endometrium analyzed by WB. Total lysate from samples were blotted and probed with an anti-LH-R specific antibody. The top bands, weighting 85 kDa refer to the mature forms of the receptor, the bottom bands weighting 75 kDa refer to the immature forms of the receptor. Reprobing of the membrane with anti-Tubulin antibody is reported in the bottom panel. (B) Immunohistochemistry for the LH/hCG-R protein on four representative EC specimens (panels 1–4) and a testicular biopsy as a positive control (inset).

significant correlation between *LH/hCG-R* mRNA expression and the LH-induced increase in cell invasiveness.

Using the Sybr Green technique we showed that the amount of *LH/hCG-R* expressed in primary EC varied from $4.67 \, e^{-02}$ to $2.36 \, e^{+03}$. This finding agrees with previous data obtained with TaqMan Real-time PCR [21], hence confirming that Real-Time PCR is a good and reliable method to detect and quantify the *LH/hCG-R* expression in endometrial tissues.

The receptor was properly translated and expressed in EC cells. This was demonstrated by both WB and IHC experiments. The latter showed that almost 65% of the cases are positive. Collectively, these results confirmed the presence of the LH/hCG-R in endometrial adenocarcinomas, as previously reported by Rao et al. [20] and by our group [12].

Previous results from our group [12], showed the relevance of the pathway centred on LH binding to its receptor on EC cells in the regulation of a relevant aspect of tumour progression, i.e. the invasion of surrounding tissues. These results were obtained in a moderately differentiated EC cell line, Hec1A, as well as on a few EC primary samples; in both cases the capability of invading in response to LH addition was dependent on PKA activation [12]. We here provide evidence that in a large number of primary EC samples the LH-induced cell invasion *in vitro* positively correlates with the amount of *LH/hCG-R* mRNA, while no correlation emerged between the amount of the receptor and other clinical–pathological characteristics of the patients.

On the whole, our data, along with those showing that LH/hCG regulates VEGF secretion [22], tend to configure the LH/hCG gonadotropin as a progression factor in EC, contributing to regulate cell invasion and angiogenesis, and hence ultimately leading to metastatic spread. Results here presented shed light to reconcile the conflicting results present in the literature, about the clinical effect of LH analogues in the treatment of recurrent or metastatic EC. We in fact report that only 35% of patients showed a high expression of *LH/hCG-R* mRNA. Interestingly, only these patients respond to exogenous recombinant LH

addition by increasing the cell invasion through Matrigel. This could in turn imply that only such patients could receive benefits from a therapy aimed at decreasing LH levels, for example, through Gn-RH analogues. We hence prospect to analyze herein all the IV stage patients (whose 5 year survival is



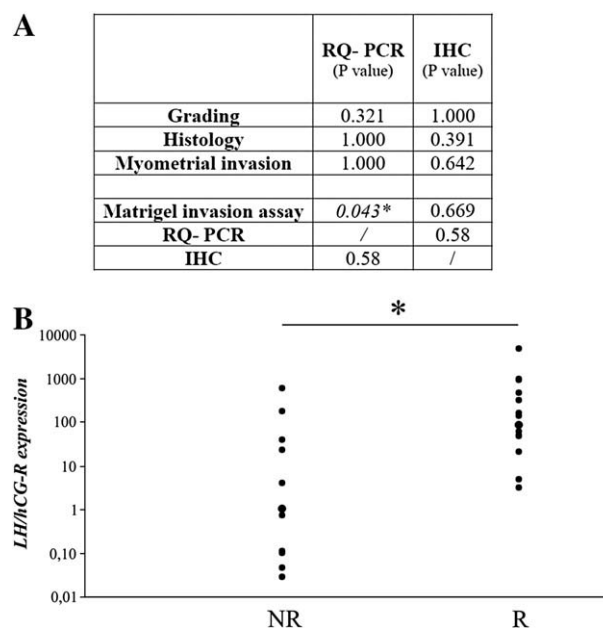| | RQ- PCR (P value) | IHC (P value) |
|---|---|---|
| **Grading** | 0.321 | 1.000 |
| **Histology** | 1.000 | 0.391 |
| **Myometrial invasion** | 1.000 | 0.642 |
| | | |
| **Matrigel invasion assay** | *0.043** | 0.669 |
| **RQ- PCR** | / | 0.58 |
| **IHC** | 0.58 | / |

Fig. 3. Correlation between *LH/hCG-R* expression and features of EC samples. (A) Correlation between LH/hCG-R expression (both RQ-PCR and IHC data) and clinical features (post-operative histology, grading and myometrial infiltration) of EC primary samples. Data were analyzed through the Fisher's exact test. In the last line the statistical analysis of data reported in panel B is shown. For the analysis of the correlation between RQ-PCR and IHC data two different RQ-PCR groups (transcript expression ≤0, transcript expression >0) and two different IHC groups were identified. (B) *LH/hCG-R* expression in non-responder (NR) and responder (R) samples. EC population was divided into two groups, one with a null (or very low) response to LH (≤1.2 fold) (NR) and the other displaying a significant response to LH addition (R) (original data are reported in Table 1). Median value NR group: $1.40 \, e^{+00}$; median value HR group: $7.42 \, e^{+01}$. Each circle represents an EC sample.

less than 10%) for the expression of LH/hCG mRNA and treat only the high expressors with Gn-RH analogues.

**Conflict of interest statement**

The authors have no conflicts of interest to declare.

## Acknowledgment

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