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Miceli *et al*

Expression and subcellular localization of CRH and its Receptors in Human Endometrial Cancer

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Key Words: Corticotropin-Releasing Hormone, CRH-receptors type 1 and type 2, Human Endometrial Carcinoma, Estrogen Receptor, Progesterone Receptor.

Abstract

CRH and its receptors are expressed in human normal endometrial cells, where they are associated to anti-proliferative progesterone-like activity. We aimed to investigate CRH, CRH-R1 and CRH-R2 expression and intracellular localization in human endometrial cancers and their relationships with tumor biological parameters.

Surgical specimens were obtained from 51 untreated endometrial cancer patients and immunohistochemistry for CRH, CRH receptors, ER, PR and Ki-67 was performed.

We found a diffuse cytoplasmic staining in 100%, 92 % and 60.7 % of tumor specimens for CRH, CRH-R1 and R2, respectively. At variance with tumor tissues, the surrounding normal endometrial glands exhibit a typical paranuclear/apical pattern for CRH and stained for CRH-R2 at the nuclear level, whereas CRH-R1 staining was similar to that observed in tumor area. Positive correlations were found between CRH-R1 and PR expression, as well as between CRH-R2 cytoplasmic pattern and more advanced FIGO stage disease, respectively.

1. Introduction

Endometrial adenocarcinoma is currently the most frequent malignancy of the female genital tract in developed countries and represents the seventh leading cause of death for cancer in women (Jemal A et al., 2008). At present, FIGO stage, depth of myometrial invasion and grade of differentiation are commonly used to predict clinical outcome and plan treatment modalities in endometrial cancer patients (Hoskins et al., 2000). However, epidemiologic observation shows 10% of stage I and 17% of stage II patients experience recurrence of disease (Pettersen 1994), thus suggesting that, apart from the above mentioned parameters, biological markers more strictly related to tumor cell biology might provide additional useful information in order to improve patients' prognostic characterization and possibly individualize treatments.

In this context, attention has been focused on corticotropin- releasing hormone (CRH), a 41-amino acid peptide originally characterized as a hypothalamic factor mediating behavioral, autonomic and neuroendocrine responses to stressors. The biological actions of CRH and related peptides (urocortins 1-3) are mediated through the activation of two CRH receptor isoforms, namely CRH-receptor type 1 (CRH-R1) and type 2 (CRH-R2), each exhibiting distinct functions, tissue-specific expression patterns and binding affinities toward CRH and other endogenous ligands (Hillhouse et al., 2006). CRH-R1 and its endogenous ligands, CRH and urocortins, were also localized in human normal endometrial tissues, where the peptide was found to exert anti-proliferative, progesterone-like effects (Ferrari et al. 1995; Di Blasio et al., 1997; Florio et al., 2002, 2006). Evidence from our group shows that similar actions are also exerted by CRH on a human endometrial tumor cell line, Ishikawa cells (IK), by the activation of CRH-R1 subtype (Graziani et al., 2002, 2006).

Apart from endometrial cancer, CRH has been shown to possess growth inhibitory effects in the vast majority of tumor cells and tissues (Melzig 1994, Graziani et al. 2007, Minas et al., 2007), through the activation of CRH-R1 subtype (Graziani et al., 2002, Reubi et al., 2003, Graziani et al., 2007). Putative anti-tumor effects of CRH also appear to be mediated by CRH-R2, via an indirect mechanism involving the down-regulation of vascular endothelial growth factor (VEGF), and the subsequent inhibition of tumor vascularization (Bale et al., 2002).

In this study, we used immunohistochemistry to investigate the expression and intracellular localization of CRH and its two receptor subtypes in endometrial cancers as well as normal surrounding tissues, in a single institutional series of primary untreated endometrial cancer patients. Correlation among morphological findings, clinico-pathological features and clinical outcome was evaluated. In particular, the reciprocal relationships among CRH, its receptors, and biological parameters such as estrogen receptor (ER), progesterone receptor (PR), and the proliferation marker Ki-67 have been analyzed.

2. Methods

2.1. Subjects

This study was conducted on 51 primary untreated endometrial cancer patients admitted, treated and followed-up at the Gynecologic Oncology Unit, Catholic University of Rome. In our Institution a written informed consent is routinely requested to patients for collection of their clinical data, as well as paraffin embedded sections for research use. Staging was performed according to FIGO classification (1988). Median age was 61 years (range 29-85). Forty (78.4%) patients were stage I-II, and 11 (21.6%) patients were stage III-IV disease. Most of the tumors (88.2%) were endometrioid adenocarcinomas and varied from well (23.5%) through moderate (49.0%) to undifferentiated tumors (27.5%). The other clinico-pathological characteristics are listed in Table 1.

All patients were submitted to total abdominal or modified radical hysterectomy *plus* bilateral salpingo-oophorectomy. At time of laparotomy fluid samples were obtained for cytologic examination and thorough intra-abdominal and pelvic exploration with biopsy or removal of any suspicious lesion was performed. Complete cytoreduction was achieved in advanced cases. Systematic pelvic and para-aortic lymphadenectomy was performed in 40 (78.4%) high risk patients (deep myometrial invasion, cervical involvement, special histotypes) according to a national randomized clinical protocol undergoing in our Department; lymph node sampling was performed in 6 cases because of visible or palpable enlarged lymph nodes. High risk patients underwent external radiotherapy or 4 to 6 cycles of cisplatin-based (each cycle: 50-75 mg/m²) chemotherapy 2-3 weeks after primary surgery. After completing treatment, patients were followed by gynecological examination, transvaginal/ abdominopelvic ultrasonography, and radiological investigations, if necessary, every 4 months for the first two years, every 6 months from the third up to the fifth year and yearly thereafter.

2.2. Immunohistochemistry

Immunohistochemistry for CRH, CRH-R1 and CRH-R2 expression was performed on 3 µm paraffin tissue sections mounted on poly-l-lysine-coated slides and dried at 37 °C overnight. After the slides were deparaffinized in xylene and rehydrated conventionally, the endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 10 min. and subjected to heat-induced epitope retrieval in microwave oven using the DAKO ChemMate detection Kit (DAKO, Glostrup, Denmark), according to the manufacturer's instructions. Endogenous biotin was saturated by a biotin blocking kit (Vector Laboratories, Burlingame CA, USA).

Cells expressing CRH, CRH-R1 and CRH-R2 were identified after 1 h incubation at room temperature by using the polyclonal goat anti-CRH antibody (C-20; sc-1759, Santa Cruz, CA, USA; dilution 1:50) recognizing a peptide mapping at the carboxy-terminus region of human CRH, the polyclonal goat anti-CRH-R1 antibody (V-14; sc-12381, Santa Cruz, CA, USA; dilution 1:50) recognizing a peptide mapping within an internal region of human CRH-R1, and the polyclonal goat anti-CRH-R2 antibody (N-20; sc-1826, Santa Cruz, CA, USA; dilution 1:50) recognizing a peptide mapping at the amino-terminous region of human CRH-R2.

CRH, CRH-R1 and CRH-R2 detections were performed by the LSAB plus System-HRP (DakoCytomation, Carpinteria, CA, USA), 30 min at room temperature, using diaminobenzidine as chromogen (DAB substrate System, DAKO). To reduce non specific binding, the sections were pre-incubated with 20% normal swine serum for 30 min at room temperature. Human trophoblast tissue was used as positive control, since human placenta has been shown to express CRH as well as both CRH receptor subtypes (Wetzka et al., 2003, Gao et al., 2008). Human placenta was obtained from a healthy subject undergoing elective caesarean section at term of gestation before the onset of labor.

ER, PR, and Ki-67 immunostainings were performed by incubating the sections for 1 h at room temperature with anti-ER (clone 1D5, recognizing ER-alfa; dilution 1:50; Ylem, Rome, Italy), anti-PR (clone 1A6, recognizing PR-A; dilution 1:50; Ylem) and anti-ki-67 (clone MIB-1; dilution 1:100;

DAKO) monoclonal antibodies. ER, PR, and Ki-67 detections were performed by the LSAB-2 System HRP (DakoCytomation).

Slides from all cases were simultaneously processed for immunohistochemistry on the TechMate Horizon automated staining system (DAKO). Negative controls were performed by omitting primary antibodies.

2.3. Evaluation of immunohistochemical staining

The analysis of all tissue sections was performed by two of the authors (FOR, EM) without any prior knowledge of clinical and biological parameters. In case of disagreement, sections were submitted to a joint evaluation by a multiheaded microscope.

CRH, CRHR-1 and CRH-R2 expressions were evaluated by considering the localization (cytoplasmic and/or nuclear), as well as the percentage of cells exhibiting immunoreaction. ER and PR stainings were scored in a minimum of 300 histologically identified neoplastic cells, showing nuclear reaction. As previously reported (Sivridis et al., 2001), tumors with ER or PR positive nuclear staining in more than 5% of tumor cells were defined as ER or PR positive, respectively. Scoring of Ki-67 was expressed as the percentage of cells with positive nuclear staining. Tumors with high Ki-67 staining in more than 40% (median value) tumor cells were considered as positive.

2.4. Statistical analysis

Fisher's exact test or χ^2 test were used to analyze the distribution of cases considered positive for the biological parameters examined according to several clinico-pathological features. Disease Free Survival (DFS) was calculated from the date of diagnosis to the date of relapse or the date last seen. Recurrence was considered as any documented relapse of the tumor either locally in the pelvis or systemic dissemination. Medians and life tables were computed using the product-limit estimate by the Kaplan and Meier method (Kaplan & Meyer 1958) and the log-rank test was employed to assess the statistical significance (Mantel 1966). Statistical analysis was carried out using SOLO (BMDP Statistical Software, Los Angeles, CA).

3. Results

3.1. CRH, CRH-R1 and CRH-R2 immunostaining

Normal glandular endometrial tissues surrounding the tumor display a typical paranuclear/apical pattern of expression (APE) for CRH (Figure 1, panel A). At variance with normal tissues, a diffuse cytoplasmic staining was observed within the tumor cells in all cases (Figure 1, panel B), although this diffuse-staining pattern was not associated to the totality of cells in the tissue, since a percentage of tumor cells maintained an APE pattern (Figure 1, Panel C). Therefore, the percentage of cells presenting with an apical pattern over the total was evaluated according to a score including: 0 (no pattern), 1 (APE <10%), 2 (10% < APE < 50%) and 3 (APE ≥ 50%). All cases with a score 2 or 3 (i.e. with APE >10%) were classified as CRH APE-positive; this occurred in 20 cases (39.2%).

Thirty-seven out of 51 specimens could be assessed for CRH-R1 staining analysis. A diffuse cytoplasmic staining with dot-like reinforcement in the basal cell domain was observed for CRH-R1 in 34 tumor tissues (Figure 2, panel B); a similar but less intense pattern was observed in the normal surrounding tissue (Figure 2, panel A).

Interestingly, CRH-R2 staining in normal endometrial glands was found to be localized at the nuclear level (Figure 3, panel A). The same pattern was also observed in 9 cases of tumors (17.6%) (Figure 3, panel C), whereas a diffuse moderate/strong cytoplasmic staining was observed in 31 tumor cases (60.7%) (Figure 3, panel B).

Placental tissue, used as positive control, stained for CRH (Fig 1 Panel D), CRH-R1 (Fig 2 Panel C), and CRH-R2 (Fig 3 Panel D) in the trophoblast cell layer.

3.2. Correlation with clinico-pathological and biological parameters

As shown in Table 2, no significant difference was documented in the distribution of CRH-APE positivity according to any of the parameters examined. Table 3 shows that a higher percentage of CRH-R1 positive cases was observed in the progesterone receptor (PR)-positive compared to PR-negative subgroup (96.8% versus 60%; $p < 0.05$); a similar trend was observed in the ER-positive versus ER-negative subgroup (96.6% versus 71.4%, respectively), although such difference did not reach the statistical significance. As far as CRH-R2 expression is concerned (Table 2), a higher percentage of CRH-R2 cytoplasmic positive cases was observed in more advanced stage disease compared to FIGO stage I tumors (81.8% versus 44.8%; p value < 0.01). No statistically significant association was found between nuclear CRH-R2 positivity and the parameters examined.

3.3. Survival analysis

As of April 2008, the median follow-up was 51 months (range= 8-82). During the follow-up period, recurrence of disease were observed in 5/29 (17.2%) early stage cases, and in 12/51 (23.5%) cases in the whole population. There were no significant differences in DFS according to CRH-APE, CRH-R1, cytoplasmic or nuclear CRH-R2 status (data not shown).

4. Discussion

To our knowledge, this is the first study evaluating the expression and intra-cellular localization of CRH and its receptors in endometrial cancers; the peculiar pattern of expression observed here

strongly suggests a role for CRH receptors and their endogenous ligands in the biology of this malignancy. Moreover, the expression and intra-cellular localization of the two CRH receptor subtypes, CRH-R1 and -R2 has been found associated with specific tumor features such as PR positive status and advanced stage of disease, respectively.

Beyond hypothalamus, CRH has been found to be expressed in other sites of the nervous system, in the gastrointestinal tract (Suda et al., 1984), vasculature (Simoncini et al., 1999), skin (Slominski et al., 1998), as well as in normal human ovaries (Mastorakos et al., 1994) and endometrium (Makriganakis et al., 1995, Mastorakos et al., 1996, Di Blasio et al. 1997, Florio et al., 2003). Moreover, during pregnancy the placenta becomes the major source of CRH, which seems to play a role in the timing of birth (Smith 1999). CRH expression was also found in different human epithelial neoplasia such as thyroid (Scopa et al., 1994), lung (Moody et al., 1994), skin (Slominski et al., 1998), and breast (Ciocca et al., 1990) cancers. As far as endometrial cancer is concerned, evidence is limited to the finding of CRH expression in Ishikawa cell line (Makriganakis et al., 1995). Despite such huge amount of studies addressed to localize CRH expression in normal and tumor parenchymas, no study has been devoted so far to investigate the patterns of intra-cellular CRH distribution.

In this study, we found that CRH is expressed in endometrial cancer cells according to two peculiar cytoplasmic patterns: diffuse and polarized. The diffuse staining of CRH, found in all tumor cases, can be considered as a morphological alteration that might reflect a deregulated intra-cellular trafficking of the protein associated to the neoplastic transformation. Conversely, the apical pattern of expression (CRH-APE), as observed in normal endometrial glands and, to a variable extent, in a discrete proportion of cancer cells, is likely to reflect a morphological aspect of CRH secretion process associated to normal cell polarity. Therefore, it would be interesting to investigate whether such polarized pattern of localization could be associated with a less aggressive tumor phenotype.

In normal human endometrium, the constitutive expression of both CRH and its R1-type receptor (particularly in stroma cells) suggests an autocrine/paracrine action of CRH on endometrial cells, where the peptide elicits progesterone-like cell differentiation (Tang et al., 1993, Ferrari et al., 1995, Makriganakis et al., 1995, Di Blasio et al., 1997, Telgmann et al., 1997, Florio et al., 2003). As an extension of the physiological role in normal endometrium, in Ishikawa endometrial tumor cells CRH is secreted and acts locally to inhibit basal and estradiol-stimulated cell growth, as well as to promote differentiation via the activation of R1 receptors and the subsequent increase in adenylyl cyclase activity (Graziani et al., 2002, 2006). Thus, one would expect that the expression of CRH-R1 receptors in human tumors is associated to a phenotype close to the physiological one. Similar to previous findings by other groups (Di Blasio et al., 1997, Karteris et al., 2004), we found that CRH-R1 is expressed in the cytoplasm of normal epithelial endometrial cells. Most importantly, CRH-R1 expression was found in a higher percentage of tumors expressing progesterone receptors, indicating that CRH-R1 expression is associated to a more differentiated and less aggressive phenotype. On the same line of evidence, progestins have been found to stimulate the expression and production of endometrial CRH through a cAMP-dependent mechanism (Makriganakis et al., 1999); in stromal endometrial cells, CRH may mediate the decidualizing effect of progesterone via the binding and activation of CRH-R1 receptors (Makriganakis et al., 2004).

Apart from CRH-R1, CRH anti-tumor activity was also found to be associated to the activation of CRH-R2 receptor subtype, through a mechanism involving VEGF and reduced tumor vascularization (Bale et al., 2002). Thus, it was interesting to investigate the expression and localization of such receptor subtype in our paradigm. Human endometrial cells were shown to express CRH-R2 under physiological conditions (Karteris et al., 2004); in the present study, we could confirm CRH-R2 expression in normal endometrial tissues surrounding the tumor; moreover, we observed an unprecedented nuclear localization, whose functional meaning remains to be established. Conversely, the expression of CRH-R2 in the tumor cell cytoplasm has been more frequently observed in stage II-IV compared to stage I tumors, thus suggesting that high CRH-R2 might identify poor prognosis tumors.

In conclusion, our immunohistochemical investigation first described the comparison between tumor and the surrounding normal tissues in terms of expression and sub-cellular localization of CRH and its receptors; on this regard, normal endometrial tissue is characterized by a paranuclear/apical pattern of expression of CRH, by the presence of CRH-R1 receptors, and the presence and nuclear localization of CRH-R2 receptors. Moreover, our preliminary observations suggest that CRH-R1 is

associated with PR positivity, thus suggesting a potential role of this receptor in the characterization of less aggressive tumors, while the varying subcellular localization of CRH-R2 receptors deserves further investigations.

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References

- Bale, T.L., Giordano, F.J., Hickey, R.P., Huang, Y., Nath, A.K., Peterson, K.L., Vale, W.W., Lee, K.F., 2002. Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization. *Proceedings of the National Academy of Sciences of the United States of America* 99, 7734-7739.
- Ciocca, D.R., Puy, L.A., Fasoli, L.C., Tello, O., Aznar, J.C., Gago, F.E., Papa, S.I., Sonego, R., 1990. Corticotropin-releasing hormone, luteinizing hormone-releasing hormone, growth hormone-releasing hormone, and somatostatin-like immunoreactivities in biopsies from breast cancer patients. *Breast Cancer Research and Treatment* 15, 175-184.
- Di Blasio, A.M., Pecori Giraldi, F., Viganò, P., Petraglia, F., Vignali, M., Cavagnini, F., 1997. Expression of corticotropin-releasing hormone and its R1 receptor in human endometrial stromal cells. *The Journal of Clinical Endocrinology and Metabolism* 82, 1594-1597.
- Ferrari, A., Petraglia, F., Gurside, E., 1995. Corticotropin releasing factor decidualizes human endometrial stromal cells in vitro. Interaction with progestin. *The Journal of Steroid Biochemistry and Molecular Biology* 54, 251-255.
- Florio, P., Arcuri, F., Ciarmela, P., Runci, Y., Romagnoli, R., Cintorino, M., Di Blasio, A.M., Petraglia, F., 2002. Identification of urocortin mRNA and peptide in the human endometrium. *J. Endocrinol.* 173, R9-R14.
- Florio, P., Rossi, M., Sigurdardottir, M., Ciarmela, P., Luisi, S., Viganò, P., Grasso, D., Fiore, G., Cobellis, L., Di Blasio, A.M., Petraglia, F., 2003. Paracrine regulation of endometrial function: interaction between progesterone and corticotropin-releasing factor (CRF) and activin A. *Steroids* 68, 801-807.
- Florio, P., De Falco, G., Leucci, E., Torricelli, M., Torres, P.B., Toti, P., Dell'Anna, A., Tiso, E., Santopietro, R., Leoncini, L., Petraglia, F., 2006. Urocortin expression is down-regulated in human endometrial carcinoma. *J. Endocrinol.* 190, 99-105.
- Gao, L., Lu, C., Xu, C., Tao, Y., Cong, B., Ni, X., 2008. Differential Regulation of Prostaglandin Production Mediated by CRH Receptor Type 1 and Type 2 in Cultured Human Placental Trophoblasts. *Endocrinology* 149, 2866-2876.
- Graziani, G., Ferrandina, G., Pozzoli, G., Vergati, M., Muzi, A., Legge, F., Tentori, L., Scambia, G., Navarra, P., 2006. Corticotropin-releasing hormone receptor-1 in human endometrial cancer. *Oncology Reports* 15, 375-379.
- Graziani, G., Tentori, L., Muzi, A., Vergati, M., Tringali, G., Pozzoli, G., Navarra, P., 2007. Evidence that corticotropin-releasing hormone inhibits cell growth of human breast cancer cells via the activation of CRH-R1 receptor subtype. *Molecular and Cellular Endocrinology* 264, 44-49.
- Graziani, G., Tentori, L., Portarena, I., Barbarino, M., Tringali, G., Pozzoli, G., Navarra, P., 2002. CRH inhibits cell growth of human endometrial adenocarcinoma cells via CRH-receptor 1-mediated activation of cAMP-PKA pathway. *Endocrinology* 143, 807-813.
- Hillhouse, E.W., Grammatopoulos, D.K., 2006. The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. *Endocrine Reviews* 27, 260-286.
- Hoskins, W.J., Perez, C.A., Young, R.C., 2000. *Principles and Practice of Gynecologic Oncology*. Lippincott Williams and Wilkins Philadelphia 1005-1007.
- Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., Thun, M.J., 2008. Cancer statistics. *CA: a Cancer Journal for Clinicians*. 58, 71-96.

- Kaplan, E., Meyer, P., 1958. Non parametric estimation from incomplete observations. *Journal of the American Statistical Association* 53, 457-481.
- Karteris, E., Papadopoulou, N., Grammatopoulos, D.K., Hillhouse, E.W., 2004. Expression and signalling characteristics of the corticotrophin-releasing hormone receptors during the implantation phase in the human endometrium. *Journal of Molecular Endocrinology* 32, 21-32.
- Makrigiannakis, A., Margioris, A.N., Chatzaki, E., Zoumakis, E., Chrousos, G.P., Gravanis, A., 1999. The decidualizing effect of progesterone may involve direct transcriptional activation of corticotrophin-releasing hormone from human endometrial stromal cells. *Molecular Human Reproduction* 5, 789-796.
- Makrigiannakis, A., Zoumakis, E., Kalantaridou, S., Chrousos, G., Gravanis, A., 2004. Participation of maternal and fetal CRH in early phases of human implantation: the role of antalarmin. *Current Drug Targets. Immune, Endocrine and Metabolic Disorders* 4, 75-78.
- Makrigiannakis, A., Zoumakis, E., Margioris, A.N., Theodoropoulos, P., Stournaras, C., Gravanis, A., 1995. The corticotropin-releasing hormone (CRH) in normal and tumoral epithelial cells of human endometrium. *The Journal of Clinical Endocrinology and Metabolism* 80, 185-189.
- Mantel, N., 1966. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemotherapy Reports* 50, 163-170.
- Mastorakos, G., Scopa, C.D., Kao, L.C., Vryonidou, A., Friedman, T.C., Kattis, D., Phenekos, C., Rabin, D., Chrousos, G.P., 1996. Presence of immunoreactive corticotropin-releasing hormone in human endometrium. *The Journal of Clinical Endocrinology and Metabolism* 81, 1046-1050.
- Mastorakos, G., Scopa, C.D., Vryonidou, A., Friedman, T.C., Kattis, D., Phenekos, C., Merino, M.J., Chrousos, G.P., 1994. Presence of immunoreactive corticotropin-releasing hormone in normal and polycystic human ovaries. *The Journal of Clinical Endocrinology and Metabolism* 79, 1191-1197.
- Melzig, M.F., 1994. Corticotropin releasing factor inhibits proliferation of AtT-20 cells. *In vitro Cellular & Developmental Biology. Animal* 30A, 741-743.
- Minas, V., Rolaki, A., Kalantaridou, S.N., Sidiropoulos, J., Mitrou, S., Petsas, G., Jeschke, U., Paraskevaidis, E.A., Fountzilas, G., Chrousos, G.P., Pavlidis, N., Makrigiannakis, A., Minas, V., Rolaki, A., Kalantaridou, S.N., Sidiropoulos, J., Mitrou, S., Petsas, G., Jeschke, U., Paraskevaidis, E.A., Fountzilas, G., Chrousos, G.P., Pavlidis, N., Makrigiannakis, A., 2007. Intratumoral CRH modulates immuno-escape of ovarian cancer cell through FasL regulation. *British Journal of Cancer* 97, 637-645.
- Moody, T.W., Zia, F., Venugopal, R., Korman, L.Y., Goldstein, A.L., Fagarasan, M., 1994. Corticotropin-releasing factor stimulates cyclic AMP, arachidonic acid release, and growth of lung cancer cells. *Peptides* 15, 281-285.
- Pettersson, F., 1994. Annual report on the results of treatment in gynaecologic cancer. FIGO Vol 22 Stockholm, Sweden.
- Reubi, J.C., Waser, B., Vale, W., Rivier, J., 2003. Expression of CRF1 and CRF2 receptors in human cancers. *The Journal of Clinical Endocrinology and Metabolism* 88, 3312-3320.
- Scopa, C.D., Mastorakos, G., Friedman, T.C., Melachrinou, M., Merino, M.J., Chrousos, G.P., 1994. Presence of immunoreactive corticotropin releasing hormone in thyroid lesions. *The American Journal of Pathology* 145, 1159-1167.
- Simoncini, T., Apa, R., Reis, F.M., Miceli, F., Stomati, M., Driul, L., Lanzone, A., Genazzani, A.R., Petraglia, F., 1999. Human umbilical vein endothelial cells: a new source and potential target for corticotrophin releasing factor. *The Journal of Clinical Endocrinology and Metabolism* 84, 2802-2806.

- Sivridis, E., Giatromanolaki, A., Koukourakis, M., Anastasiadis, P., 2001. Endometrial carcinoma: association of steroid hormone receptor expression with low angiogenesis and bcl-2 expression. *Virchows Archiv : an International Journal of Pathology* 438, 470-477.
- Slominski, A., Ermak, G., Mazurkiewicz, J.E., Baker, J., Wortsman, J., 1998. Characterization of corticotropin-releasing hormone (CRH) in human skin. *The Journal of Clinical Endocrinology and Metabolism* 83, 1020-1024.
- Smith, R., 1999. The timing of birth. *Scientific American* 280, 68-75.
- Suda, T., Tomori, N., Tozawa, F., Mouri, T., Demura, H., Shizume, K., 1984. Distribution and characterization of immunoreactive corticotropin-releasing factor in human tissues. *The Journal of Clinical Endocrinology and Metabolism* 59, 861-866.
- Tang, B., Guller, S., Gurpide, E., 1993. Cyclic adenosine 3',5'-monophosphate induces prolactin expression in stromal cells isolated from human proliferative endometrium. *Endocrinology* 133, 2197-2203.
- Telgmann, R., Maronde, E., Taskén, K., Gellersen, B., 1997. Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. *Endocrinology* 138, 929-937.
- Wetzka, B., Sehringer, B., Schäfer, W.R., Biller, S., Hör, C., Benedek, E., Deppert, W.R., Zahradnik, H.P., 2003. Expression patterns of CRH, CRH receptors, and CRH binding protein in human gestational tissue at term. *Experimental and Clinical Endocrinology & Diabetes* 111, 154-161.

FIGURE LEGENDS

Figure 1. CRH immunostaining in endometrial adenocarcinoma. Paranuclear/apical pattern of CRH expression (APE) in normal endometrial tissue surrounding the tumor (A), diffuse cytoplasmic CRH staining observed in all tumor tissues (B), tumor area with APE pattern (C). Placental tissue was used as positive control for CRH immunostaining (D).

Figure 2. CRH-R1 cytoplasmic immunostaining in normal endometrial tissue surrounding the tumor (A); a diffuse cytoplasmic staining with dot-like reinforcement was observed for CRH-R1 in most of endometrial adenocarcinoma analyzed (B). Placental tissue was used as positive control for CRH-R1 immunostaining (D).

Figure 3. CRH-R2 immunostaining in endometrial adenocarcinoma. Specific CRH-R2 nuclear staining in normal endometrial glands (A), diffuse cytoplasmic CRH-R2 staining observed in most of tumor tissues (B), tumor case with CRH-R2 nuclear pattern (C). Placental tissue was used as positive control for CRH-R2 immunostaining (D).

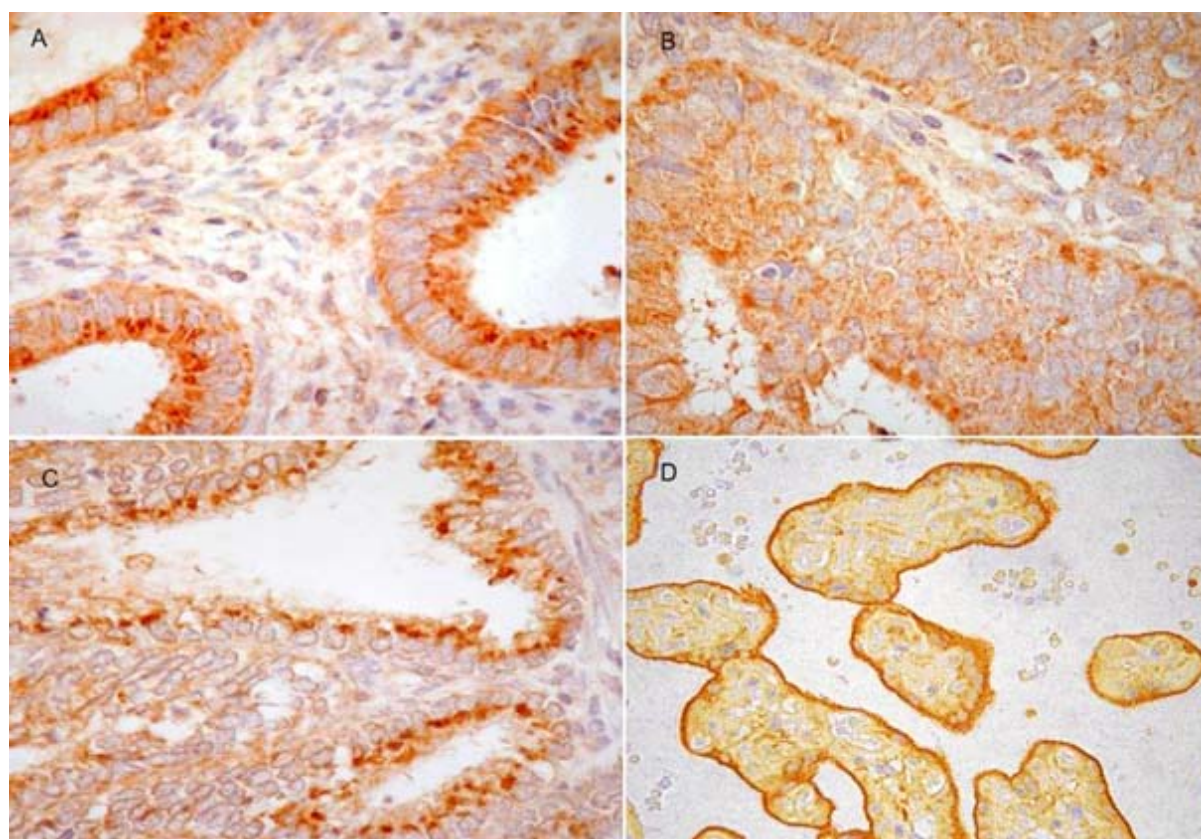
Figure 1.

Figure 2.

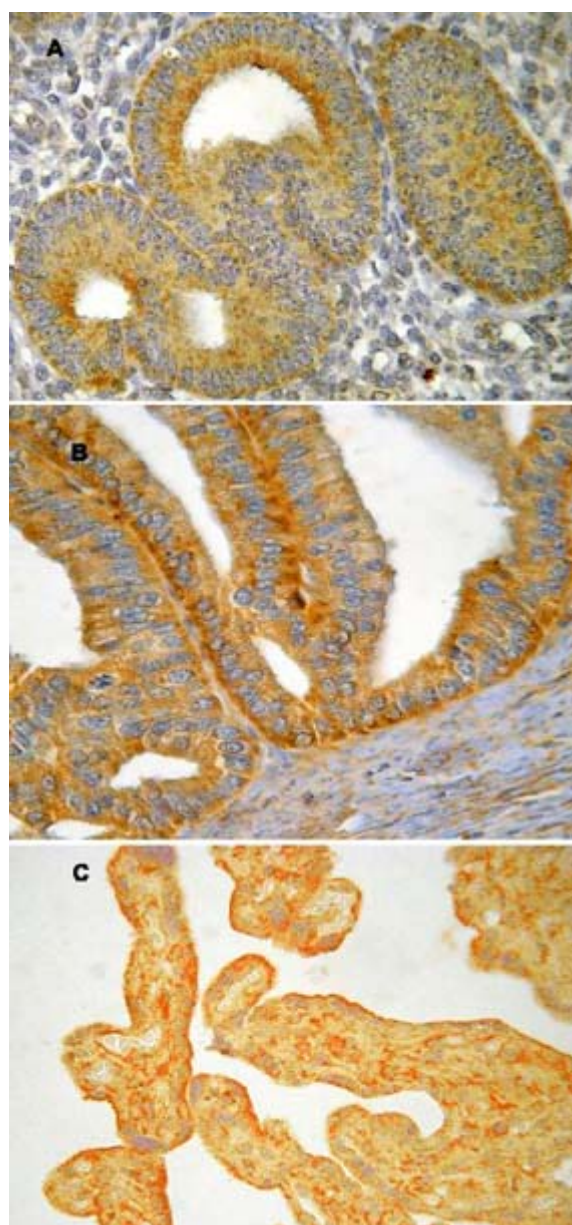


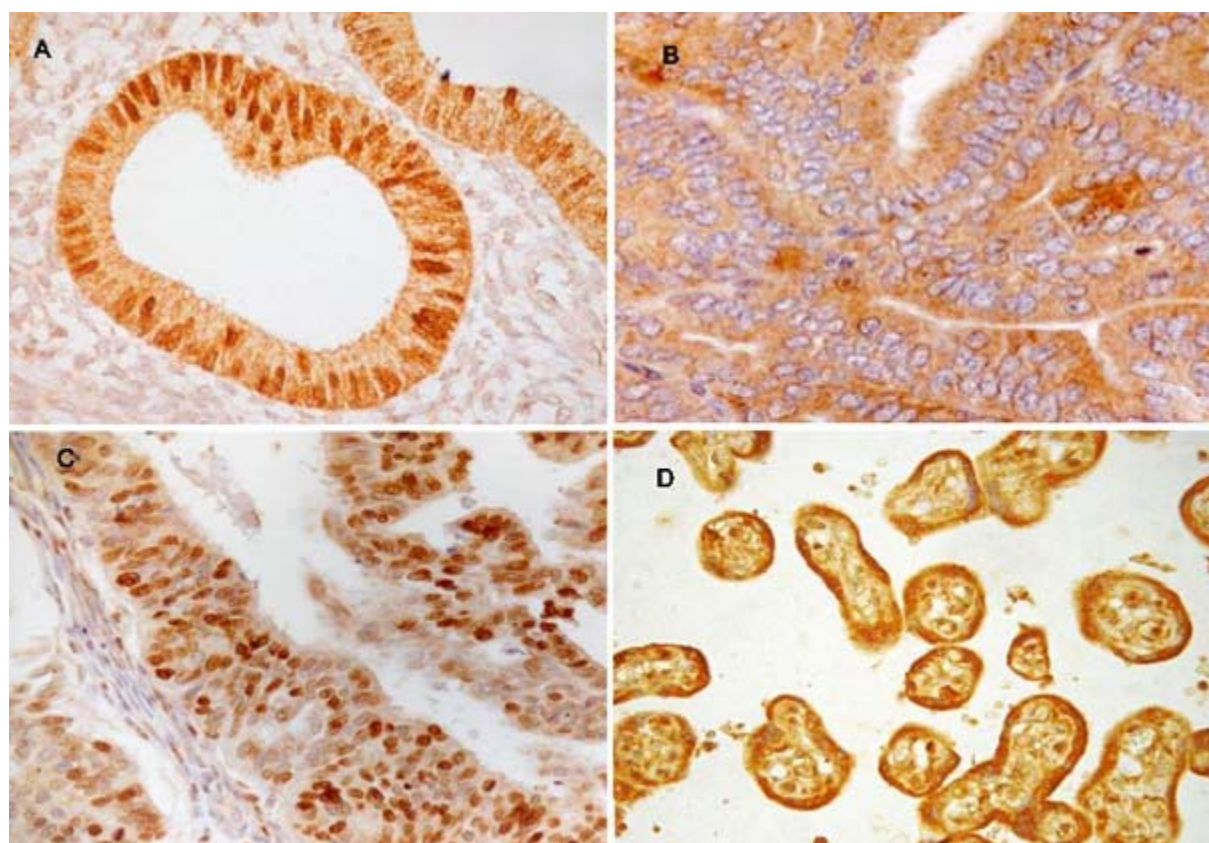
Figure 3.

Table 1. Clinico-pathological and biological features of our sample series.

Features	N°. of cases (%)
All cases	51
Age	
< 60	24 (47.1)
≥ 60	27 (52.9)
Stage	
I	29 (56.8)
II	11 (21.6)
III-IV	11 (21.6)
Histotype	
Endometrioid	45 (88.3)
Serous	3 (5.9)
Clear cell	2 (3.9)
Undifferentiated	1 (1.9)
Grade	
1	12 (23.5)
2	25 (49)
3	14 (27.5)
Lymph node status	
Negative	31 (60.7)
Positive	9 (17.7)
n.a.	11 (21.6)
ER	
Positive	36 (70.6)
Negative	15 (29.4)
PR	
Positive	42 (82.3)
Negative	9 (17.7)
Ki-67	
Low	30 (58.8)
High	21 (41.2)

Table 2. Distribution for CRH-APE, CRH-R1, cytoplasmic and nuclear CRH-R2 positivity according to clinico-pathological parameters.

Features	N°. of cases	CRH APE Positivity N°. (%)	Cytoplasmic CRH-R2 positivity N°. (%)	Nuclear CRH-R2 positivity N°. (%)
All cases	51	20 (39.2)	31 (60.7)	9 (17.6)
Age				
< 60	24	12 (50.0)	16 (66.7)	4 (16.7)
≥ 60	27	8 (29.6)	15 (55.5)	5 (18.5)
Stage				
I	29	10 (34.5)	13 (44.8) ** ^a	6 (20.7)
II	11	6 (54.5)	10 (90.9)	2 (18.2)
III-IV	11	4 (36.4)	8 (72.7)	1 (9.1)
Histotype				
Endometrioid	45	18 (40)	27 (60.0)	9 (20)
Serous	3	0	3 (100)	0
Clear cell	2	1 (50)	1 (50)	0
Undifferentiated	1	1 (100)	0	0
Grade				
1	12	4 (33.3)	7 (58.3)	2 (16.7)
2	25	11 (44.0)	16 (64.0)	4 (16.0)
3	14	5 (35.7)	8 (57.1)	3 (21.4)
Lymph node status				
Negative	31	15 (48.4)	19 (61.3)	7 (22.6)
Positive	9	2 (22.2)	7 (77.8)	1 (11.1)
n.a.	11	-	-	-
ER				
Positive	36	16 (44.4)	21 (58.3)	7 (19.4)
Negative	15	4 (26.7)	10 (66.7)	2 (13.3)
PR				
Positive	42	18 (42.9)	27 (64.3)	9 (21.4)
Negative	9	2 (22.2)	4 (44.4)	0
Ki-67				
Low	30	12 (40.0)	18 (60.0)	5 (16.7)
High	21	7 (33.3)	13 (61.9)	4 (19.3)

** Indicates significant P-values <0.01 calculated by Fisher's exact test for proportion.

^a FIGO stage I versus stage II-IV.

Table 3. Distribution for CRH-R1 positivity according to clinico-pathological parameters.

Features	N°. of Cases	CRH-R1 Positivity N°. (%)
All cases	37	34 (92)
Age		
< 60	15	15 (100)
≥ 60	22	19 (86.3)
Stage		
I	25	23 (92)
II	7	7 (100)
III-IV	5	4 (80)
Histotype		
Endometrioid	36	34 (92)
Serous	1	0
Clear cell	-	-
Undifferentiated	-	-
Grade		
1	9	8 (88.8)
2	20	19 (95)
3	8	7 (87.5)
Lymph node status		
Negative	20	18 (90)
Positive	7	6 (85.7)
n.a.	10	
ER		
Positive	30	29 (96.6)
Negative	7	5 (71.4)
PR		
Positive	32	31 (96.8) *
Negative	5	3 (60)
Ki-67		
Low	22	21 (95.4)
High	15	13 (86.6)

* Indicates significant P-values <0.05 calculated by Fisher's exact test for proportion.