

# Epiregulin as a marker for the initial steps of ovarian cancer development

ABRAHAM AMSTERDAM<sup>1</sup>, ELIAS SHEZEN<sup>2</sup>, CALANIT RAANAN<sup>3</sup>, YASMIN SLILAT<sup>1</sup>,  
ALON BEN-ARIE<sup>4</sup>, DIANA PRUS<sup>5</sup> and LETIZIA SCHREIBER<sup>6</sup>

Departments of <sup>1</sup>Molecular Cell Biology, <sup>2</sup>Immunology and <sup>3</sup>Biological Services, The Weizmann Institute of Science; <sup>4</sup>Kaplan Medical Center, Rehovot 76100; <sup>5</sup>Department of Pathology, University Hospital, Hadassah Ein Kerem, Jerusalem 911210; <sup>6</sup>Wolfson Hospital, Holon 58100, Israel

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**Abstract.** Epiregulin (Ep) was found to be produced in non-cancer ovarian cells in response to gonadotropin stimulation as well in ovarian cancer cells in an autonomous manner. However, there were no systematic follow-up studies of Ep expression in the development of different stages of ovarian cancer. Using specific antibodies to Ep and the indirect immunocytochemistry methods, we found that in normal ovary the staining for Ep was mainly confined to the epithelial cells, while the stromal cells were only occasionally and moderately stained. In contrast in benign serous and mucinous tumors most of the tumor cells showed a clear staining in the cytoplasm. In borderline serous and mucinous tumors the staining was much more intensive, and appear occasionally in aggregated form. In serous, mucinous and endometrioid carcinomas labeling remain high, with more frequent aggregated form. It is suggested that follow-up of the expression of Ep can serve as a reliable early indication of the development of ovarian cancer. Moreover, the cytoplasmic aggregation of Ep may suggest a specific mechanism of the release of this growth factor to the extracellular space in order to exert its autocrine and paracrine effect on the family of the EGF receptors.

## Introduction

Ovarian cancer is the most lethal gynecological cancer in women because early detection is extremely difficult (1-14) and because it is resistant to most chemotherapeutic drugs (15-17). The average lifespan after detection is 40.8 months for serous cancer, 21.3 months for clear cell carcinomas, 17.6 months for mucinous carcinomas and 50.9 months for endometrioid ovarian carcinomas (11). Amphiregulin (Ar) and Ep, being EGF-like family members, confer binding specificity also for the ERBB

4 receptors (18,19). Ar and its receptor, epidermal growth factor receptor (EGFR), were expressed in a series of invasive ductal breast carcinoma specimens (20). Ep was found to be involved in the stimulation of tyrosine phosphorylation of ERBB-4 and EGFR in human breast carcinoma cell lines (21).

Ep exerts a mitotic activity in various primary cell types, such as rat hepatocytes (22), as well as in various types of human cancer cell lines, especially in epithelial tumor cell lines (22). In 1991 Johnson *et al* (23) demonstrated expression of Ar in ovarian carcinoma, and the localization of endogenous Ar in the nucleus. Immunohistochemical detection of EGF gene family was also demonstrated in ovarian carcinogenesis. In 1995 Gordon *et al* (25) demonstrated mRNA phenotyping of the major ligands and receptors of EGF gene family in human epithelial cancer cell lines. It was demonstrated that knockout of heparin-binding EGF-like growth factor can be a potential method to attenuate cancer progression (reviewed in refs. 26,27).

Transforming growth factor  $\alpha$ , Ar and crypto-1 are frequently expressed in advanced human ovarian carcinoma (28). Expression of epidermal growth factors was demonstrated in endometrial cancer (29). Recently, an association of Ep expression in tumorigenesis through activation of Ki-Ras-signalling pathway has been suggested in human colon cancer cells (30). Likewise, Ep was found to be upregulated and stimulated growth of human pancreatic cancer cells (31).

Ep and Ar were found to be expressed in the normal ovary during the cycle (32,33). These growth factors bind to EGFR, ERBB2 or ERBB4 receptors leading to phosphorylation of the receptor (34,35) and activation of ERK 1/2 by the dual phosphorylation catalysed by MAPKK and MAPKKK (36-40). This MAPK cascade, which is one of the major intracellular signaling pathways, plays a key role in proliferation, differentiation and apoptosis (36-40). In quiescent cells, MEK acts as a cytoplasmic anchor of ERK 1/2, and when activated by stimulation, ERK 1/2 is phosphorylated on the threonine and tyrosine residues, which is then dissociated from MEK. This dissociation is necessary for nuclear translocation of ERK 1/2 (36-40). Upon stimulation, the nuclear localization of pERK 1/2 is required, for example for changes in neurite outgrowth in PC12 cells, and the transformation of fibroblasts (reviewed in refs. 41,42).

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*Correspondence to:* Dr Abraham Amsterdam, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel  
E-mail: abraham.amsterdam@weizmann.ac.il

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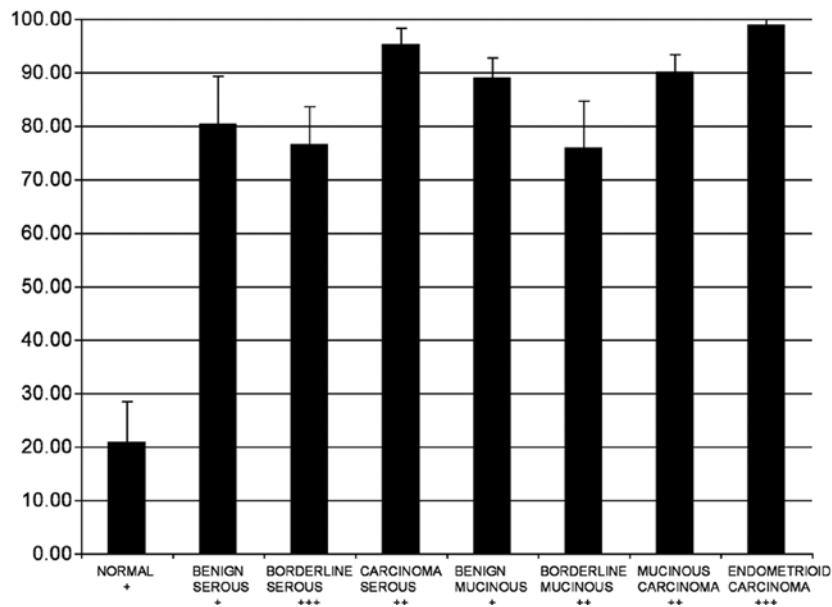


Figure 1. Percentage of labeled cells with antibodies to Ep at different stages of tumor development and carcinogenesis (stage II) of serous, mucinous and endometrioid cancer. The difference between normal ovary, benign serous borderline tumors as well as carcinomas is significant ( $p < 0.000013$ ). The differences between normal ovaries, mucinous tumors and carcinomas is highly significant ( $p < 0.0001$ ), as well as for endometrioid carcinomas compared to normal ovary ( $p < 0.000004$ ). +, intensity of labeling.

There have been many attempts to cure ovarian cancer with poor success, using antibodies to EGFR. However, recently monoclonal antibodies cetuximab and panitumab have established efficacy as single agent and in combination with chemotherapy in advanced colorectal cancer and lung carcinomas (43,44). In addition, high mRNA levels of EGF ligands; Ep and Ar have been associated with increased responsiveness to cetuximab (reviewed in ref. 45).

Recently, genetic and pharmacological analysis identified a set of genes such as EGFR ligand Ep, cyclooxygenase-2 (COX2) and the metalloproteinase-1 and -2, that have been found to be associated with metastasis in a variety of cancers (46). It was, therefore, suggested that inhibition of EGFR and COX2 could minimize the development and metastasis of these cancers (46).

As for the ovarian cancer, there is no follow-up of the appearance of Ep, and the progression of tumor development, although it is evident that ovarian cancer cells produce EGF-like factors (reviewed in ref. 47). In the present work, we demonstrate that the high levels of Ep is already evident in benign tumors and borderline tumors. The early detection of this growth factor can greatly assist in selecting the proper therapy as early as possible. Thus, the lifespan of women suffering from ovarian cancer, that is presently considered as a lethal disease, could be improved.

## Materials and methods

**Materials.** Monoclonal antibodies to p53 (clone 421) were kindly donated by Professor M. Oren of our Institute. Antibodies to epiregulin were goat anti-human epiregulin affinity purified, R&D Systems. Second specific antibodies conjugated to HRP were from N-Histofine, Japan.

**Procedures.** Localization of Ep was performed by the indirect immunostaining method of ovarian sections, formaldehyde-fixed, and paraffin-embedded specimens (3,48-50), obtained

from women at the age of 41-79, through several hospitals in Israel. For control, sections of normal ovaries were obtained from women aged of 41-73. Specimens of paraffin sections were obtained from 11 normal ovaries and 8-11 ovaries of benign, borderline and carcinomas of each group. As for endometrioid cancer, we obtained only 2 carcinoma specimens. All carcinomas were at stage II. Permissions for the research were obtained from Helsinki Committees of each hospital: Kaplan Medical Center, Rehovot, Israel, Hadassah University Hospital, Ein Kerem Jerusalem, Israel, and the Wolfson Hospital, Holon, Israel. Staining of sections with anti-p53 were performed using specific antibodies, followed by staining with secondary specific antibodies conjugated to HRP against the primary antibodies.

**Microscopic examination.** Immunostained sections and parallel sections stained with hematoxylin and eosin were observed in Lietze or Nikon microscopes, using x10-x1000 magnification. For scoring the number of labeled cells and nuclei in normal and different stages of the ovarian tumor development, pictures were taken at magnification x1000. Eight to twelve different areas of stained slides of each different stage of ovarian tumor development (and controls of normal ovaries) were photographed and the number of labeled nuclei and total nuclei were scored, calculating the percentage of labeled nuclei in each photograph, and the mean value  $\pm$  SD of all fields were calculated. At each stage of the ovarian tumors development (and normal tissue) total of ~950 nuclei were scored.

**Statistical analyses.** Statistical evaluation of the different percent of labeled nuclei among the different stages of ovarian tumor development was performed, including control of normal ovary. Analysis of variance (ANOVA) followed by multiple comparison tests or Student's t-test as appropriate was performed. Calculations were performed using SPSS software (Student's t-test, Version II, Chicago IL, USA). Values of  $p < 0.05$  were considered statistically significant.

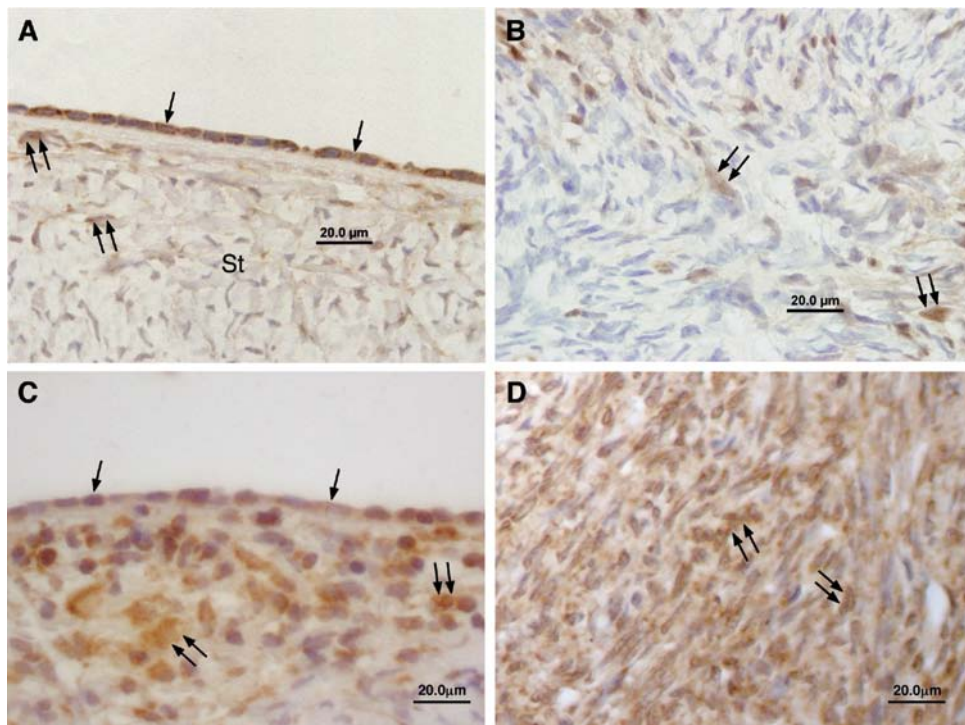


Figure 2. Intensity of staining normal ovary. (A) The layer of epithelial cells is clearly labeled with antibodies to Ep (arrows). Some labeling is evident occasionally in stromal cell (double arrows). (B) Labeling is also evident only occasionally in the bulk of the stromal cells (double arrows). (C) Another normal ovary (according to the classification using straight morphology). The epithelial layer is clearly labeled, with antibodies to Ep (arrows). Stromal cells are also clearly and densely labeled (double arrows). (D) The labeling is intense also in the bulk of the stromal cells (double arrows). It is therefore suggested to be considered of high potential to develop a tumor and was not scored as normal ovary in our statistic evaluation of staining. Original magnification x1000.

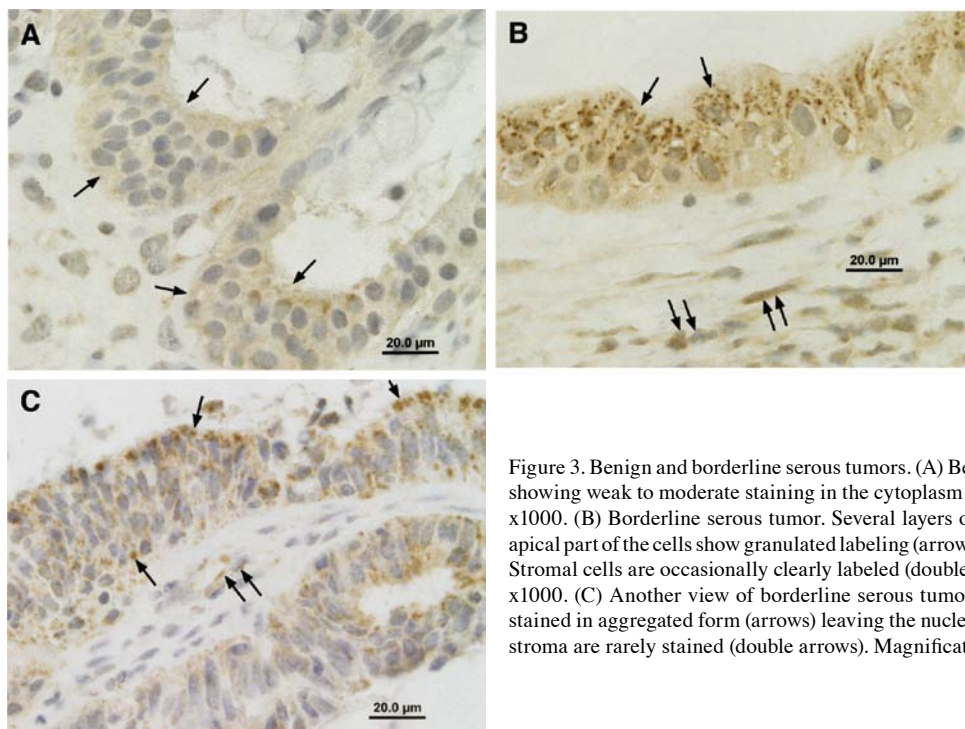


Figure 3. Benign and borderline serous tumors. (A) Benign serous tumor cells showing weak to moderate staining in the cytoplasm (arrows). Magnification x1000. (B) Borderline serous tumor. Several layers of cells are labeled. The apical part of the cells show granulated labeling (arrows) with antibodies to Ep. Stromal cells are occasionally clearly labeled (double arrows). Magnification x1000. (C) Another view of borderline serous tumor. Cytoplasm of cells is stained in aggregated form (arrows) leaving the nuclei unstained. Cells of the stroma are rarely stained (double arrows). Magnification x1000.

## Results

In order to verify whether Ep can serve as an early marker for the development of ovarian cancer, the following steps were taken.

*Selection of antibodies.* We have selected the R&D antibodies for the staining of Ep since, among six companies this was the

only antibody that could be blocked by pre-incubation with x20 concentration in molar ratio of Ep prior to the staining of the sections (data not shown).

*Normal ovary.* We have analyzed specimens of normal ovary, characterized by straight morphology, following H&E staining and staining with antibodies to EP, and found that the labeling (Figs. 1 and 2A) with Ep antibodies was confined mainly to the

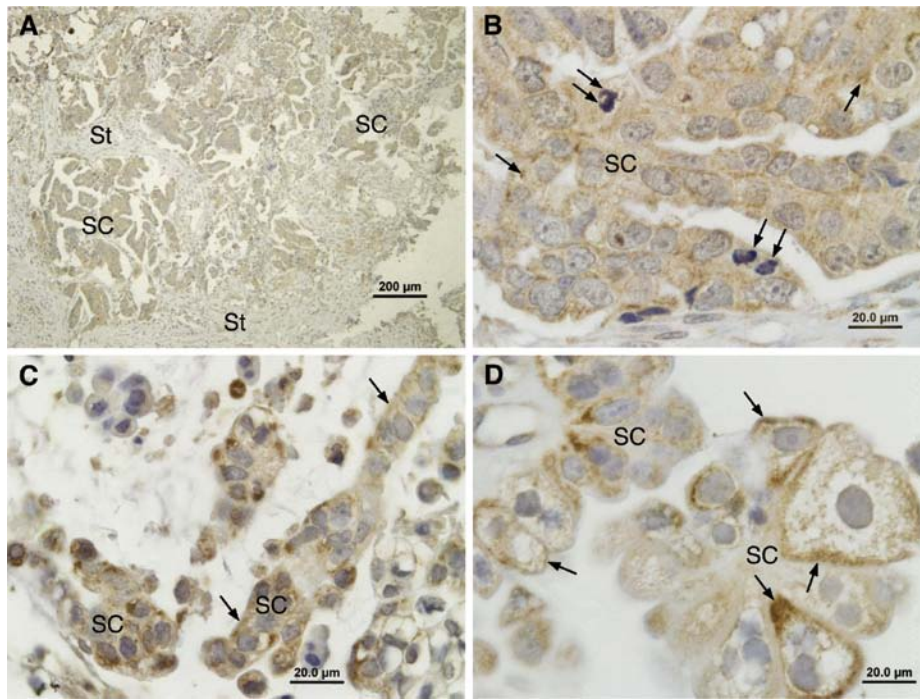


Figure 4. Serous carcinoma. (A) Low magnification. Islet of cluster carcinoma cells are clearly labeled with antibodies to Ep (SC). Stromal cells are faintly stained (St). (B) Serous carcinoma cells at high magnification (SC). Cytoplasm of cells is clearly stained occasionally in aggregates (arrows), leaving the nuclei unstained. Mitotic figures are evident. Cells in metaphase and anaphase are visible (double arrows). Magnification x1000. (C) A different view of serous carcinoma (SC). Cytoplasm of carcinoma cells is more homogeneously stained than in B (arrows). Magnification x1000. (D) Another different view of serous carcinoma (SC). Nuclei and cell bodies are much larger. Staining is confined to the circumference of cells (arrows). Magnification x1000.

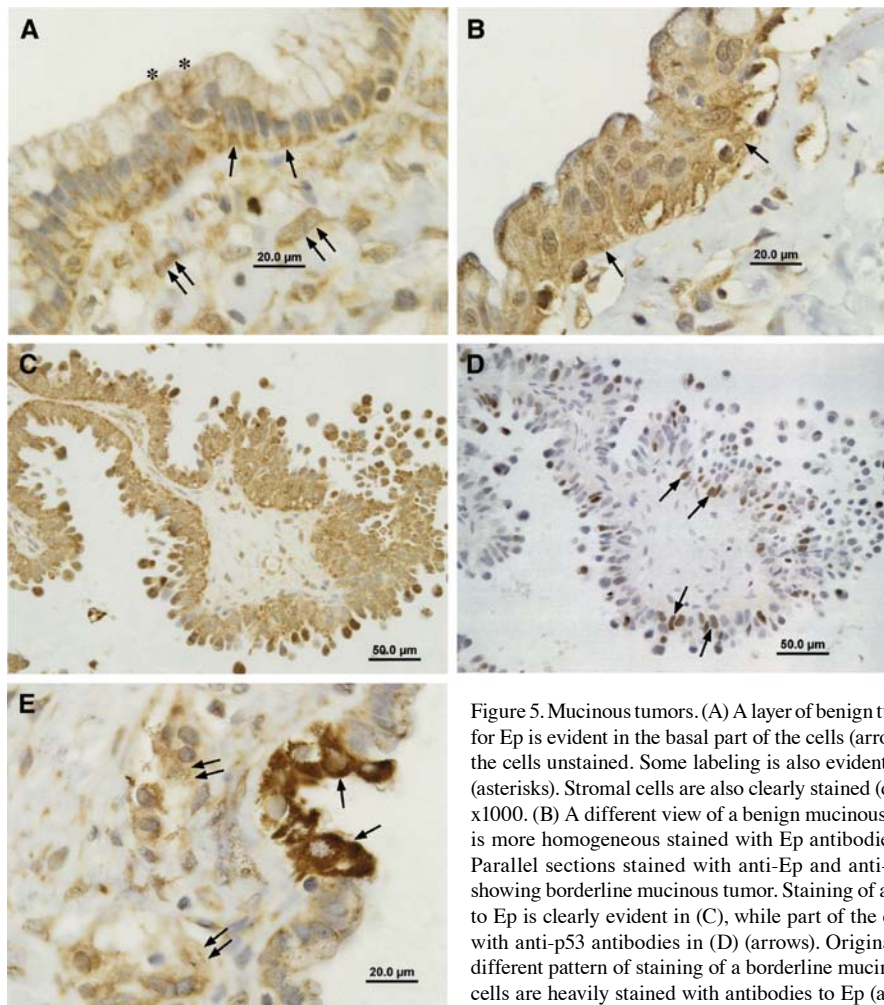


Figure 5. Mucinous tumors. (A) A layer of benign tumor elongated cells. Staining for Ep is evident in the basal part of the cells (arrows) leaving the main body of the cells unstained. Some labeling is also evident at the upper part of the cells (asterisks). Stromal cells are also clearly stained (double arrows). Magnification x1000. (B) A different view of a benign mucinous tumor. Tumor cell cytoplasm is more homogeneously stained with Ep antibodies (arrows) x1000. (C and D) Parallel sections stained with anti-Ep and anti-p53 antibodies respectively, showing borderline mucinous tumor. Staining of all tumor cells with antibodies to Ep is clearly evident in (C), while part of the cell nuclei are clearly stained with anti-p53 antibodies in (D) (arrows). Original magnification x400. (E) A different pattern of staining of a borderline mucinous tumor. Clusters of tumor cells are heavily stained with antibodies to Ep (arrows) while the neighboring stromal cells are clearly stained as well (double arrows). Magnification x1000.

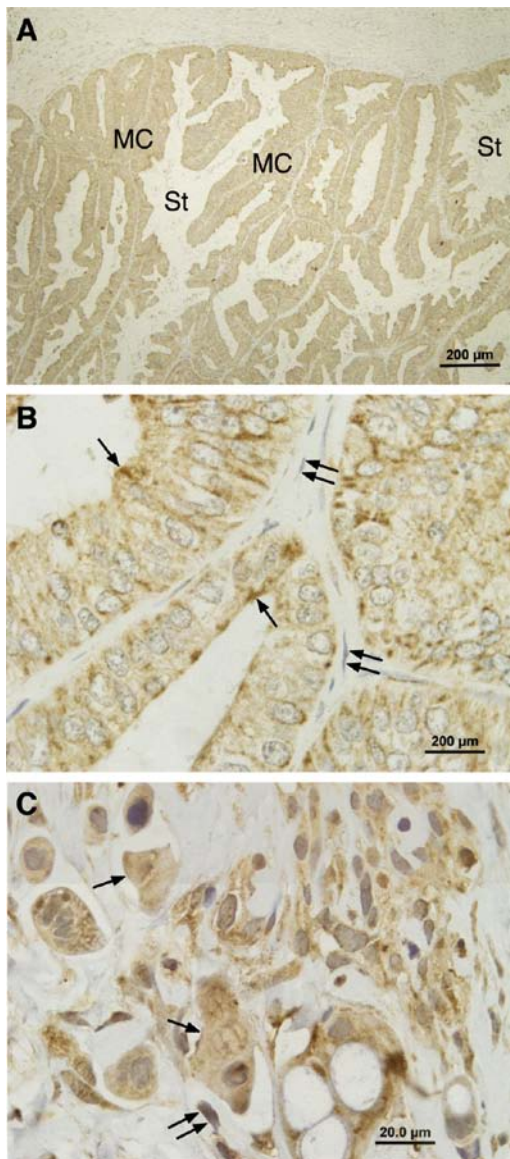


Figure 6. Mucinous carcinomas. (A) At low magnification carcinoma cells, organized in a specific pattern, are clearly labeled with antibodies to Ep (MC). Stromal cells are only weakly labeled (St). Magnification x100. (B) At high magnification cancer cells are organized in a specific array. Cells are clearly labeled with aggregates of Ep, mainly at the circumference of the cells (arrows). Stroma cells remained essentially unstained (double arrows). Magnification x1000. (C) Different appearance of mucinous carcinoma cells. Cancer cells are less organized than in (B) and the labeling with antibodies to Ep is more homogeneous (arrows). Magnification x1000.

epithelial cells, while stromal cells were labeled only occasionally (Fig. 2). However, closer to 50% of the normal ovary specimens (5 of 11) were widely and clearly stained with Epi antibodies (Fig. 4C and D), suggesting that Ep staining could be a good indication for initiation of tumor growth that cannot be observed by straight morphology, or using some other biomarkers. These ovaries were not scored as normal ovaries.

**Serous benign tumors.** Percentage of labeled cells increased dramatically (Figs. 1 and 3A). A weak but clear staining was evident in all layers of the tumor cells.

**Serous borderline tumors.** In borderline serous tumors there were two types of staining. The first type of staining with anti-

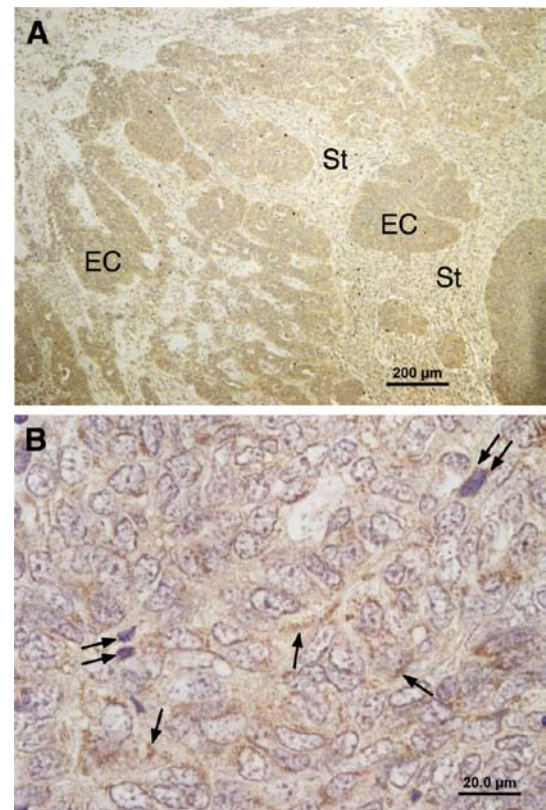


Figure 7. Endometrioid carcinoma. (A) Aggregated cancer cells are embedded in stromal cells, clearly labeled with antibodies to Ep (EC). Stromal cells are only weakly labeled (St). (B) High magnification of endometrioid cancer cells. Cells are tightly packed. Ep appears in aggregates (arrows). Mitotic images are evident (double arrows). Magnification x1000.

bodies to Ep revealed weak but clear staining in cells bordering the ovarian stroma (Fig. 3B), while the apical part of the cells was stained with distinct small aggregates. Some of the stromal cells were clearly labeled with Ep antibodies. In the other view of borderline tumors the tumor cell nuclei were smaller and Ep aggregates were present both in the apical and the basal part of the cells (Fig. 3C). Only few stromal cells were stained with anti-Ep antibodies.

**Serous ovarian carcinoma.** In serous ovarian carcinomas (stage II) islands of tumor cell aggregates were clearly evident, embedded in the ovarian stromal tissue. They were clearly stained with antibodies to Ep, while the stromal tissue remained essentially unstained (Fig. 4A). At high magnification, serous carcinomas have sometimes different appearance: the first one showed large nuclei and clear cytoplasmic staining with Ep antibodies, leaving the nuclei unstained. Only mild staining could be observed in the cytoplasm. Occasionally aggregates of stained Ep were also evident (Fig. 4B). In the second appearance of serous carcinoma the cytoplasmic staining of Ep was more intensive and more homogeneous (Fig. 4C). The third appearance was characterized by large cells with labeling mainly at the circumference of the cells leaving the nuclei unstained (Fig. 4D).

**Mucinous benign tumors.** Benign tumors showed two different appearances. The first one showed labeling with anti-Ep antibodies at the basal part of the cells and some in the apical part, leaving the rest of the cell body, including the nuclei,

unstained (Fig. 5A). Stromal cell adjacent to the tumor cells were moderately labeled with Ep antibodies. In the second appearance the entire cytoplasm of tumor cells was clearly labeled with Ep antibodies (Fig. 5B), while some stromal cells were also labeled.

**Mucinous borderline tumors.** In order to verify whether borderline tumors contain already muted p53, we stained adjacent sections one with Ep antibodies (Fig. 5C) and the second with anti-p53 antibodies (Fig. 5D). Distinct staining to p53 was evident in part of the cell nuclei indicating that mutation of p53 is already evident in borderline mucinous tumors. Additional image of borderline tumor was evident with clusters of cells heavily labeled with anti-Ep antibodies (Fig. 5E). Interestingly, prior to staining the specific tumor was classified according to the morphological appearance, at the most, as a benign tumor.

**Mucinous carcinomas.** At low magnification mucinous carcinoma show more differentiated image than serous carcinomas and was clearly stained with Ep antibodies (Fig. 6A). At high magnification, two distinct patterns of staining were revealed: the first was with an organized form. Clear labeling was mainly confined to the edges of the tumor cells (Fig. 6B). Stromal cells were essentially free of labeling. In the other image, cells were bigger and the entire cytoplasm was stained. Occasionally large cytoplasmic vacuoles were evident. Stromal cells were often stained in the cytoplasm leaving the nuclei unstained (Fig. 6C).

**Endometrioid ovarian tumors.** We obtained only two specimens of ovarian endometrial carcinomas (stage II). Low magnification showed bulks of tumor cells stained with anti-Ep antibodies. Stromal cells were weakly stained and most of them remained unstained (Fig. 7A). High magnification revealed dense appearance of cancer cells with mitotic figures. Staining with Ep antibodies mainly appeared in aggregates. Nuclei were free of labeling (Fig. 7B).

## Discussion

In the present report we clearly demonstrate that Ep can serve as a sensitive early marker for ovarian tumor development and carcinogenesis. It was earlier demonstrated by RT-PCR that there was no difference in the content of RNA between normal ovary and cancer ovary (51). However, there is no documentation on the protein level that could be affected by siRNA and possibly by various intracellular proteinases (reviewed in refs. 52-54). Moreover, high percentage of normal ovaries defined by straight morphology were found by us as already at the stage of benign or even borderline tumors. In addition, analysis of RNA on to the entire cancer tissue may not be always accurate since it may also contain residues of non-cancer tissue. Therefore, the protein levels as estimated by immunocytochemistry may be more accurate as for the level of Ep in the different stages of tumor and malignant cancer development. We found a similar situation for phosphorylated ERK 1/2 which is the outcome of activation of EGF receptor, which in turn will phosphorylate MAPK and activate the ERK 1/2 (55).

Serous carcinomas include 80% of ovarian cancer (3) while mucinous carcinomas traditionally represent 12-13% of

ovarian carcinomas. However, recently, according to a variety of biomarkers it was suggested to represent only 2-3% of total carcinomas (16). Nevertheless the different images that appeared following staining with antibodies to Ep would suggest novel subtypes of ovarian carcinomas. We estimate the amounts of Ep by the intensity of staining with anti-Ep antibodies. We avoid performing Western blot analyses since at benign, borderline tumors and carcinomas stage 2 most probably the tissues contained a significant amount of stromal cells which may blur the results. It should be noted that other members of the EGF-like member proteins such as HB-EGF and Ar may also serve as sensitive members for the development of benign, borderline and ovarian cancer as exemplified in the present work for Ep.

High magnification images revealed in the present report that Ep was often found in cytoplasmic aggregates very often at the circumference of the cells. This may suggest a specific mechanism of release (56) or shedding (57) of the growth factor without losing other essential intracellular organelles. This issue could probably be solved using high resolution EM where Ep is stained with Ep antibodies attached to colloidal gold particles, which produce a high contrast in the electron beam of the microscope (reviewed in ref. 58). It should be noted, as mentioned before, that the release of the growth factor to the extracellular space is a prerequisite to exert its mitogenic effect through the activation of MAPK and phosphorylation of ERK 1/2 which will migrate to the nucleus (36-40).

Early detection of ovarian cancer will no doubt improve the prognosis of ovarian cancer and this could be achieved by biomarkers for early detection of the disease as was exemplified in the present report for the EGF-like factor, Ep.

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