# Alpha-folate Receptor Expression in Epithelial Ovarian Carcinoma and Non-neoplastic Ovarian Tissue

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**Abstract.** *Background: The goal of this study was to evaluate the expression pattern and intracellular localization of alpha-folate receptor (α-FR) protein in human ovarian carcinoma compared with non-neoplastic ovarian tissue. Materials and Methods: Using immunohistochemistry (IHC), α-FR protein expression was analyzed in specimens of 104 human ovarian carcinomas and 30 non-neoplastic ovaries. Results: In 97% of the ovarian carcinomas, clear α-FR protein expression was detected (14% weak, 39% moderate, 44% strong). In the non-neoplastic ovaries, no (37%) or only weak (63%) expression was observed ($p<0.0001$). The tumor cells were characterized by a diffuse and homogeneous staining pattern. In tumor and non-tumor tissue, α-FR protein was detected predominantly in the cellular cytoplasm. In 41% of the ovarian carcinomas, cytoplasmic expression was localized towards the outer boarders of the invasive tumor cells and 30% exhibited additional nuclear α-FR protein expression. Conclusion: Compared with non-neoplastic ovaries, α-FR protein is overexpressed in human ovarian carcinoma tissue.*

Ovarian cancer is the third most common gynecological malignancy and the leading cause of death from gynecological cancer in the industrialized nations (5). Despite advances in the treatment of ovarian cancer, the rather low overall survival rate also reflects a limited efficacy of chemotherapeutic regimens in the case of advanced stage disease (4). In this paradigm, recently described tumorspecific molecular targets such as the alpha-folate receptor (α-FR) may enrich conventional chemotherapeutic strategies and thus improve the outcome of patients with ovarian cancer (14, 21, 26, 32).

Folic acid is an essential vitamin and of crucial importance for one-carbon transfer processes mediated by enzyme systems involved in DNA synthesis (1). Under physiological conditions, folate uptake occurs predominantly by an ATP-dependent reduced folate carrier (RFC), whereas under conditions of limited folate supply uptake occurs mainly *via* folate receptor mediated endocytosis (2, 6, 11, 29). *In vitro* and *in vivo* studies have indicated that the expression of α-FR allows epithelial tumor cells to proliferate even under conditions of limited folate supply, pointing to α-FR expression as an acquired marker of tumor cell proliferation and thus, tumor biology and patient prognosis (2, 11, 16, 17, 29, 33). Increased expression of α-FR has been described in various tumor tissues derived from human lung, intestinal tract and kidney compared with normal tissue counterparts (2, 13, 24).

Due to the systemic toxicity of cytostatic drugs, the targeted delivery of these drugs to the tumor cells is a desired goal in modern cancer chemotherapy (9, 10, 18, 26, 32). Antitumor prodrugs with receptor-affinity ligands, which are absorbed into the cells by tumor-associated receptors, may improve the effectiveness of cytostatic drugs and decrease the systemic toxicity of a given chemotherapeutic agent (9, 10, 12, 18, 26, 31, 32, 34, 36). Because of the increased expression of α-FR in epithelial tumor cells, it has been emphasized that this receptor could represent a useful target for the development of new and selective anticancer drugs (11, 14, 21, 22, 30, 32, 36). Indeed, various *in vitro* and *in vivo* studies with cell lines and xenograft models have already demonstrated increased activity, enhanced antitumor efficacy and reduced toxicity of folate receptor targeted drug conjugates compared to non-targeted controls (19, 27, 35, 38).

Some evidence for the increased expression of α-FR protein in gynecological malignancies has been reported (7, 15, 20, 23, 33, 37), but there is a paucity of data regarding

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*Key Words:* Ovarian carcinoma, alpha-folate receptor, folate receptor overexpression, targeted therapy.

Table I. *Characteristics of patients and tumors*

| | |
|---|---|
| Tumor type | |
| Ovarian carcinoma | 104 (100) |
| Age at time of diagnosis (years) | |
| Median | 64 |
| Range | 35-86 |
| FIGO stage | |
| I | 5 (4.8) |
| II | 2 (1,9) |
| III | 97 (93.3) |
| Histological grade | |
| G1 | 1 (0.9) |
| G2 | 35 (33.7) |
| G3 | 66 (63.5) |
| G4 | 2 (1,9) |

NOTE: n (%) patients for each variable.

the expression pattern and subcellular localization of α-FR protein in normal and tumor tissue of gynecological origin.

## Materials and Methods

*Patients and tissue samples*. In total, 104 patients with ovarian carcinoma (serous, n=8; serous-papillary, n=79; papillary, n=17) and 30 control patients were enrolled. All the patients were diagnosed and underwent surgery at the Department of Obstetrics and Gynecology at University Medical Center, Freiburg, Germany between 1998 and 2005. The tissue samples were retrieved from the pathology archive and the study was approved by the local Ethical Committe. Tissue arrays of tumor and control tissue were prepared and serial array sections were used for immunohistochemistry (IHC). Most of the patients were diagnosed at advanced stage disease (93% FIGO stage III). The characteristics of the patients and tumors are summarized in Table I.

*Immunohistochemistry*. IHC was performed at the Institute of Pathology, Freiburg University Medical Center. Routinely formalin-fixed and paraffin-embedded specimens were used. Serial sections (3 μm) were cut and then placed on glass slides. The sections were deparaffinized using xylol, rehydrated in alcohol series and then treated with 0.3 % $H_2O_2$ (all from Sigma, Dreisenhofen, Germany). For antigen retrieval, the sections were boiled in citrate buffer (pH 6.0) and then blocked with normal goat serum (diluted 1:10 with 1xTBS) for 15 minutes. Subsequently, the primary antibodies, rabbit-anti-human-α-FR-antibodies (kind gift of Dr. Jan Holm, Copenhagen, Denmark), were applied to the tissue sections and allowed to incubate for 60 minutes at room temperature. The optimal dilution of the primary antibodies (1:50 with TBS/BSA) and the digestion conditions was determined by a previous series of titration experiments. The sections were then washed in TBS and incubated with an appropriate biotinylated secondary antibody (1:400, goat-anti-rabbit-immunoglobulin; DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature. Following further washing in TBS, the sections were exposed to preformed avidin-biotin-horseradish peroxidase complex (DakoCytomation) for 30 minutes, washed again, and the antigen visualized by the application of 3-amino-9-ethylcarbazole (DakoCytomation) for 10 minutes. The sections were then counterstained with Mayer's hematoxylin. Sections of fallopian tube and mature human placenta were used as positive control tissues. Negative controls were performed with non-immune serum supernatant instead of the primary antibody. Specific staining of α-FR (present or absent) was evaluated using x40 magnification by light microscopy.

Table II. *Intensity of α-FR protein expression in ovarian carcinoma and non-neoplastic ovarian tissue*.

| IHC score | Controls n (%) | Tumor tissue n (%) |
|---|---|---|
| 0 | 11 (37) | 3 (3) |
| 1+ | 19 (63) | 14 (14) |
| 2+ | 0 | 41 (39) |
| 3+ | 0 | 46 (44) |

IHC: immunohistochemistry.

A semi-quantitative score was applied to classify the intensity of IHC staining as absent (0), weak (+1), moderate (+2), or strong (+3). The percentage of positive cells for each intensity was estimated for the whole of the tumor on the section. The extent of expression was simplified as focal when <50% of the cells were stained and diffuse when >50% of cells were stained. In all the samples investigated, diffuse and widespread staining of the tumor cells was observed. Therefore, the resulting IHC score was based exclusively on the intensity of IHC staining. The subcellular localization of α-FR expression was either homogenously cytoplasmic, cytoplasmic with stronger expression towards the outer cell borders, or cytoplasmic and nuclear.

*Statistical analysis*. For statistical analysis, the patients were divided into two groups, cases (ovarian carcinoma) and controls (non-neoplastic tissue), and analyzed for between-group differences in α-FR protein expression by Mann-Whitney *U*-test. *P*-values of <0.05 were considered to be statistically significant. SPSS version 14 (SPSS Inc., Chicago, IL, USA) was used for the calculations.

## Results

The intensity of α-FR protein expression in the carcinoma and non-neoplatic tissues is shown in Figure 1 and Table II.

All the positively stained non-neoplastic ovarian tissue samples revealed a homogeneous cytoplasmic expression pattern of α-FR protein in the stromal cells, with only 7% (2/30) cases showing some evidence of nuclear staining.

In contrast to the non-neoplastic tissue, α-FR protein was detected in the invasive tumor cells of almost all (97%; n=101) of the ovarian carcinoma samples and only 3% (n=3) revealed no α-FR protein expression (Figure 1 and Table II). Statistical analysis revealed a significant difference between ovarian carcinoma samples and controls with respect to α-FR protein expression ($p<0.0001$).

Interestingly, two staining patterns within the tumors became apparent: 58 (56%) of the tumor samples showed a

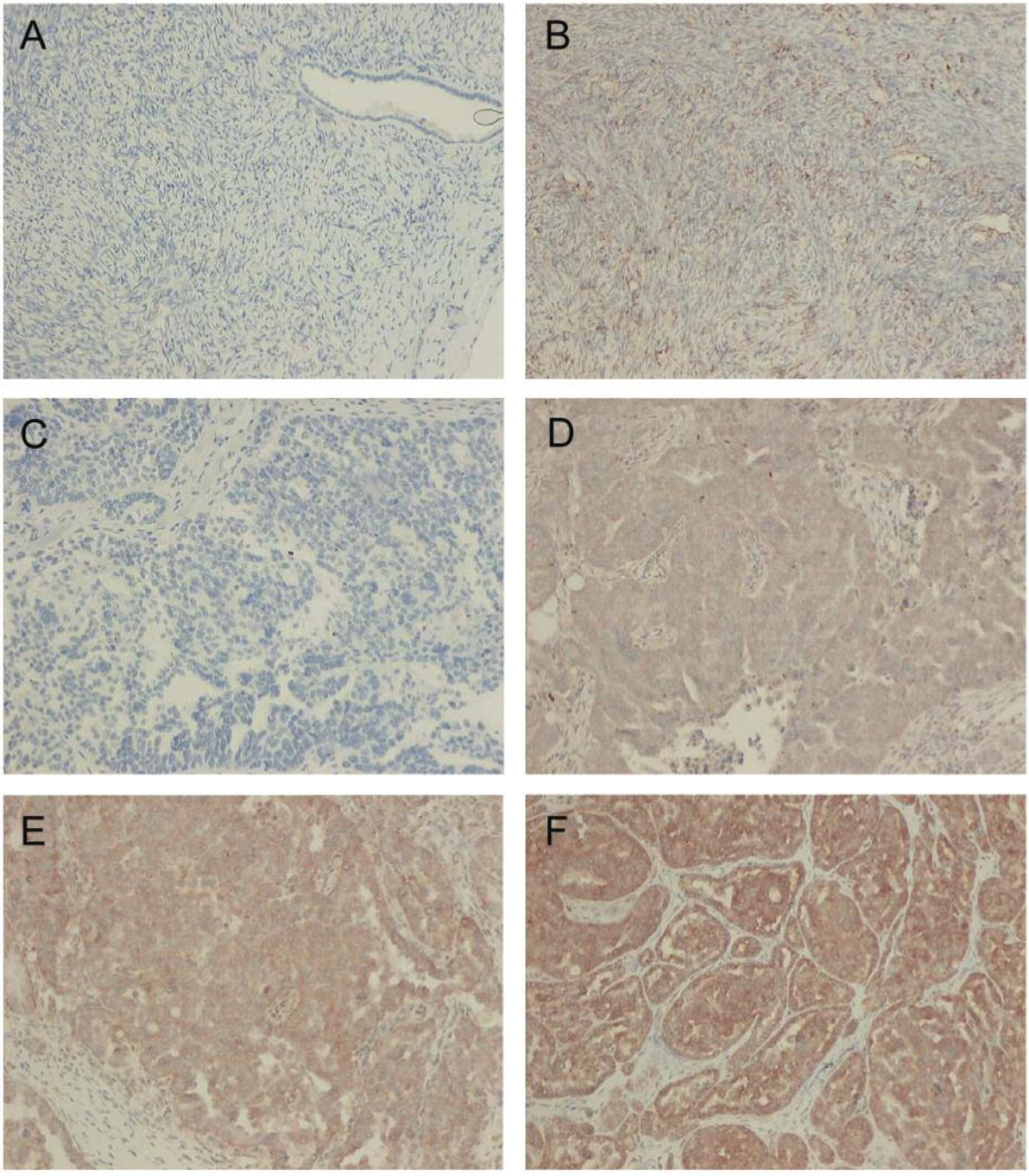


Figure 1. *Immunohistochemical analysis of α-FR protein expression in ovarian carcinoma and non-neoplastic ovarian control tissue. Non-neoplastic ovarian tissue (A and B) and ovarian carcinoma tissue (C-F). A) and C) no immunoreactivity for α-FR, B) and D) weak α-FR protein expression (score 1+), E) moderate α-FR protein expression (score 2+), F) strong α-FR protein expression (score 3+). Magnification ×100.*

homogeneous cytoplasmic staining pattern of the invasive tumor cells (Figure 2A and Table III), whereas in 43 tumor samples (41%) the α-FR expression was focally enriched towards the outer tumor cell borders (Figure 2B and Table III). In 31 ovarian carcinoma samples (30%), additional nuclear staining was observed in the invasive tumor cells (Figure 2C and Table III). There was no isolated nuclear α-FR staining in the investigated patient cohort.

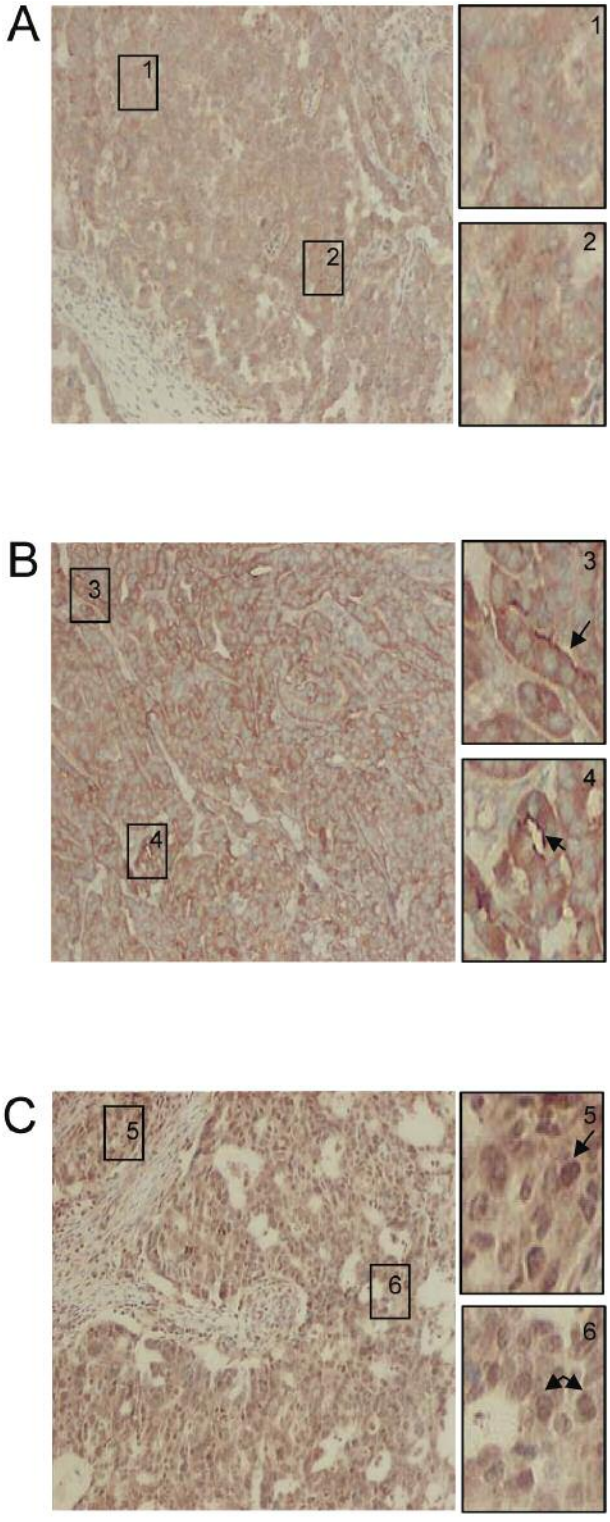


Figure 2. *Subcellular localization of α-FR protein expression in tumor cells of ovarian carcinoma tissue. A) homogenous cytoplasmic immunoreactivity, B) cytoplasmic immunoreactivity with staining directed towards the cell borders, C) cytoplasmic and nuclear immunoreactivity. Magnification ×100. Right panels each show two corresponding enlarged areas as denoted in the left panel A, B and C.*

Table III. *Staining pattern and subcellular localization of α-FR protein in ovarian carcinoma and non-neoplastic ovarian tissue.*

| Staining pattern of α-FR protein | Controls n (%) | Tumor tissue n (%) |
|---|---|---|
| Homogeneous cytoplasmic | 19 (63) | 58 (56) |
| Cytoplasmic enriched toward cell borders | 0 | 43 (41) |
| Cytoplasmic and nuclear | 2 (7) | 31 (30) |

## Discussion

The present study clearly pointed to α-FR protein overexpression in late-stage ovarian carcinomas. In contrast, α-FR protein was not or only barely expressed in non-neoplastic control tissue. Our results are in accordance with those of other studies investigating α-FR protein expression *in vitro* and *in vivo* in tumor tissue from human ovaries or cell lines using different methods (3, 13, 15, 20, 23, 24, 28, 33). Elevated *α-FR* mRNA expression has previously been described (7, 28, 37). However, some of these studies were limited by the small number of cases and/or the absence of normal ovarian tissue as a control. With IHC, Garin-Chesa *et al*. (13) and Holm *et al*. (15) investigated a small sample size of ovarian carcinoma specimens; Mantovani *et al*. (23) examined ovarian carcinoma cell lines, but no control tissue. Only Bagnoli *et al*. (3) included a larger number of ovarian carcinoma specimens (n=168) in an IHC study, but investigated only two control tissues. None of the previous IHC studies investigated the subcellular localization of α-FR protein systematically and the expression pattern of α-FR protein has therefore remained unclear to date.

Various review articles have described the α-FR as a GPI-fixed (glycosyl-phosphatidyl-inositol) cell membrane receptor which also exists in a soluble receptor form (2, 6, 11, 29). We therefore initially had expected to also detect membrane staining of the α-FR protein. However, no definitive membrane staining was identified in the invasive tumor cells of the ovarian carcinomas investigated in the present study. It may well be that the α-FR is lost as a functional membranous protein in late stages of ovarian carcinomas and may particularly be involved in early stages of tumor development. The lack of detection of membranous staining may also be related to the rapid functional translocation of the α-FR protein. After intracellular synthesis, the receptor binds to the cell membrane, resulting in cell surface expression and thus becoming available for folic acid uptake by endocytosis. After the release of folic acid into the cell, the endosome is recycled and the receptor returns to the cell surface (2, 6, 8, 11, 29). Depending on the cell type, cell cycle, cell growth and metabolism, the recycling rates differ. There is a dynamic exchange of α-FR protein between the cell surface and the

internal pool (16, 25). In the steady state, up to 50-75% of α-FR protein is located in the endosomal compartment (29). A slow recycling rate results in retention and storage of α-FR protein in the endosomal compartment and the membranes of cell organelles (1, 8, 29). This may explain the fact that in the present study on fixed tissue specimens, only homogenous intracellular cytoplasmic staining of α-FR protein was seen, without detailed visualization of short-term engagement of α-FR with specific cell organelles or the cell membrane. If α-FR protein is expressed at the same level or shuttles rapidly between the cellular compartments, the differentiation between membranous and cytoplasmic staining is not possible by simple semi-quantitative IHC. Additional methods such as 3D immunofluoresence imaging or FACS analysis, which are specific for the detection of membrane bound antigens, are needed for more precise definition. It is tempting to speculate that the detected stronger cytoplasmic immunoreactivity of α-FR protein at the tumor cell borders in 41% of the ovarian carcinoma samples might be due to receptor accumulation towards the cell membrane. In fact, α-FR internalization and thus intracellular receptor localization is necessary for receptor-mediated drug delivery of folate-bound conjugates (21, 30).

Importantly, α-FR protein was also detected in the nuclear compartment of some tumors. None of the previously published studies described such nuclear α-FR protein localization (2, 3, 6, 7, 11, 13, 23, 25, 28, 29, 33). Possibly α-FR protein in its soluble form may transport folic acid to the nucleus for DNA synthesis. This interesting new aspect was beyond the scope of the present study and should be investigated in more detail in future studies looking at the regulation of α-FR protein expression and interaction *in vitro*.

In conclusion, α-FR protein is highly and consistently overexpressed in the tumor tissue of patients with advanced stage ovarian carcinoma compared with controls. The α-FR might therefore become a potential target for selective molecular-based antitumor therapeutic strategies.

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