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*ORIGINAL ARTICLE*

# Expression of galectin-3 and carcinoembryonic antigen in the trophoblast of the gestational trophoblastic disease

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## Summary

***Purpose:*** *Galectin-3, an endogenous β-galactoside-binding protein, has been implicated in the regulation of many physiological and pathological cellular processes through specific interactions with complementary ligands. The level of galectin-3 expression has been correlated with metastatic potential in many tumor types. The present study was designed to investigate possible correlation of the expression of galectin-3 with carcinoembryonic antigen (CEA), one of the putative galectin-3 ligands, in the transformed trophoblast of the gestational trophoblastic diseases (GTDs) compared to the invasive trophoblast (interstitial and endovascular) of the normal first trimester of pregnancy placenta.*

***Materials and methods:*** *Double immunohistochemistry was used to examine paraffin sections of 9 specimens of normal placenta, 8 cases of invasive mole and 7 cases of choriocarcinoma. Immunoreactivity was scored semiquantitatively to include both the percent of trophoblast cells stained as well as the intensity of staining.*

***Results:*** *Data presented here demonstrate similar patterns of expression for galectin-3 and CEA in transformed GTDs trophoblastic cells, as well as in normal invasive trophoblast. Upregulation of galectin-3 in transformed GTDs trophoblast was followed by high levels of CEA expression in the same tissue specimen (significant differences at $< 0.05$, Mann-Whitney Rank Sum Test).*

***Conclusion:*** *These results may point to the CEA as one of the ligands for galectin-3 in transformed trophoblast, which merits further investigation.*

**Key words:** CEA, galectin-3, gestational trophoblastic disease, trophoblast

## Introduction

Galectins are known as a family of lectins with specific carbohydrate-recognition moieties due to which they are implicated in specific cellular interactions occurring during cell growth, differentiation and cancer progression [1-3]. Galectin-3, one of the most widely reported galectins, previously referred to as CBP35, RL-29, L-31, L-34, low affinity IgE binding protein and Mac-2, is a ~30 kDa protein and a member of a growing family of multifunctional galactoside-binding proteins [4]. This lectin has been identified in the nucleus and cytoplasm and also attached to appropriately glycosylated cell surface and extracellular matrix ligands. The precise function of galectin-3 is not completely understood. Intracellularly, galectin-3 seems to be involved in pre-mRNA splicing, proliferation [5] and molecular control of apoptotic processes [6]. The extracellular role of galectin-3 is manifested through its ability to modify cell-cell and cell-matrix interactions by binding to complementary glycoconjugates expressed on the cell surface or in the extracellular matrix (ECM) [7]. Thus, galectin-3 has been implicated in a variety of biological func-

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tions including cell growth, proliferation, differentiation, cell adhesion, apoptosis, embryogenesis, implantation and placentation [3,4,8,9]. Galectin-3 is also expressed in many neoplastic lesions and is believed to play a role in transformation, invasion and metastasis [9-11]. Heterogeneity in galectin-3 expression has been detected in a number of human cancers including ovarian [12], breast [13], colon [14,15], thyroid [16] and gastric carcinomas [17], lymphomas [18], melanomas and fibrosarcomas [19]. An increase in galectin-3 expression is often associated with malignancy and metastatic capacity of tumor cells [15,20].

CEA has been proposed to be one of the putative ligands for galectin-3 in neoplastic cells [21], due to the specific polylactosamine sequences in its structure [22]. Human CEA is the prototypic member of a large gene subfamily of the immunoglobulin superfamily of cell surface glycoproteins [22,23] and is overexpressed in many types of human tumors, including ovarian [24], colorectal, breast and lung carcinomas. For a long time it has been one of the most prevalent tumor markers of the gastrointestinal tract and biliary duct system [25,26]. A number of functions have been ascribed to CEA including cell adhesion, ecto-ATP-ase activity, bile salt transport activity, receptor internalization, recognition of bacteria and viruses, blocking of cell differentiation and regulation of signal transduction [27].

Our recent study on galectin-3 expression in GTD demonstrated upregulation of this lectin in invasive trophoblast of all GTDs studied, while its expression in normal interstitial and endovascular trophoblast was very low or almost undetectable [28]. GTDs embrace a wide spectrum of trophoblastic diseases caused by abnormal proliferation and transformation of trophoblastic cells of the placenta. These trophoblastic disorders are characterized by great histomorphologic variability followed by differences in biological and clinical behaviour. Invasive mole and choriocarcinoma represent the malignant neoplasms, the former composed of infiltrating trophoblast and molar villi and the latter exclusively of cytotrophoblast and syncytiotrophoblast [29].

In the present study, expression of CEA in GTDs as well as its possible correlation with galectin-3 in transformed trophoblast was studied.

## Material and methods

### *Tissue samples*

This study was performed on tissue samples from 15 patients with GTDs and tissues from 9 individuals who had legal abortion for no medical reason. Tissue samples were obtained from the Institute of Obstetrics and Gynecology, Clinical Center of Serbia, Belgrade, during 1995-2004. The tissues used in this study were obtained in accordance with the local ethical standard.

Of the specimens from the patients with GTDs, 8 were invasive moles and 7 choriocarcinomas. The gestational age ranged from 5-11 weeks for invasive moles and from 6-12 weeks for choriocarcinomas. In one case of choriocarcinoma the analysed material originated from curettage. In 6 additional cases the material was obtained from hysterectomy; in 5 patients surgery was performed after initial diagnostic curettage, and in one case the initial treatment was hysterectomy. Hysterectomy material with confirmed diagnosis of invasive mole was used in all 8 cases. Samples of normal placental tissue (with placental villi, decidual and placental bed tissue) were obtained from 9 legally terminated first trimester pregnancies.

Five patients with GTDs included in this study received chemotherapy prior to surgery. One patient diagnosed with invasive mole was treated with one cycle of MAC regimen before the operation. Three patients with choriocarcinoma received 20 mg of methotrexate each, before the operation. All patients received postoperative chemotherapy according to a standard protocol (MAC, EMA/CO, methotrexate and cyclophosphamide). Formalin-fixed, paraffin-embedded tissues were drawn from the surgical pathology files of the Department of Pathology, Institute of Obstetrics and Gynecology, Clinical Center of Serbia. For this study, 20 sections per sample were used.

### *Antibodies and reagents*

Polyclonal sheep antibody against CEA was produced and characterized in our laboratory [30]. CEA was isolated from liver metastasis and the purified glycoprotein was used for the production of antibodies by immunization of sheep.

A rat monoclonal antibody to galectin-3 (M3/38, ATCC TIBB-166) against mouse macrophage cell surface antigen Mac-2 [31] which is identical to L-29 i.e. galectin-3 [32] was kindly provided by Dr M.E. Huflejt, La Jolla Institute for Allergy and Immunology, San Diego, CA. Antibody to CEA was used at 1: 4000 dilution and to galectin-3 at 1: 25 dilution. Trophoblast cells in tissue specimens were identified by immunostaining using mouse monoclonal antibody to cytokeratin-18 (DakoCytomation, Denmark) at a dilution of 1: 6000. Immunostaining was performed on 5-$\mu$m-thick sections using the avidin-biotin-peroxidase

complex (ABC) technique with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Biotinylated horse anti-mouse IgG was used for galectin-3 and cytokeratin detection and biotinylated goat anti-rabbit IgG for CEA detection (both from Vector Laboratories). Biotinylated secondary antibodies were used in our experiments at dilutions of 1: 200 in non-immune serum solution. DAB and Vector Nova RED substrate kits for peroxidase were purchased from Vector Laboratories, Burlingame, CA, USA. All other reagents were of the best commercial grade available.

*Immunohistochemical analysis*

Sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated via graded ethanol concentrations. Endogenous peroxidase activity was quenched by 30 min incubation in 1% (v/v) $H_2O_2$/ethanol followed by a wash in distilled water. Prior to staining antigen unmasking (for immunostaining with anti-cytokeratin) was performed by boiling the slides in 0,01M citrate buffer (pH 6) for 1 min. After cooling, nonspecific binding of antibodies in all sections was blocked by incubation with non-immune serum for 10 min at room temperature. For statistical evaluation, slides were stained for cytokeratin, galectin-3 and CEA in single immunostaining procedure. In order to gain better insight into the precise distribution of CEA and galectin-3 at the cell level, double immunostaining experiments were performed. The sections were first incubated with primary anti-galectin-3 antibody for 1h at room temperature, then for 30 min with corresponding biotinylated secondary antibody. This was followed by incubation with avidin-biotinylated peroxidase complex (ABC) for 30 min. The reaction was visualized using a solution of DAB/Ni as chromogen. Between steps, sections were washed 3 times with PBS. After the first cycle of immunohistochemistry, slides were washed in PBS and then incubated with anti-CEA antibody at 4° C overnight. The corresponding biotinylated secondary antibody and ABC complex were applied as described above. The reaction was visualized using a solution of Vector Nova Red as chromogen. In addition, single immunostaining experiments were performed. Negative controls were performed routinely. Omission of the primary antibody or use of non-immune serum in place of a specific antibody resulted in complete absence of staining. After single immunostaining procedure, the slides were lightly counterstained with hematoxylin for 15 sec to allow morphological analysis of the placental tissues. Slides were dehydrated, cleared in xylene, mounted, examined and photographed using a Reichert-Jung microscope with a Leica DC Digital Camera System (Wetzlar, Germany).

*Immunohistochemical evaluation and statistical analysis*

Immunoreactivity was independently evaluated semiquantitatively by two investigators. Sections stained with each antibody (single immunostaining) were scored for the staining intensity as well as the percent of the trophoblast cells stained. For the staining intensity of trophoblast cells, samples were scored as follows: 0 for no reactivity, 1 for the presence of both negative and trace positive cells, 2 for weak, 3 for moderate, and 4 for strong staining. The percent of galectin-3/CEA stained trophoblast cells (identified by cytokeratin) was scored as follows: 0 for no positive cells, 1 for 1-20% cells positive, 2 for 21-50% cells positive, 3 for 51-80%, and 4 for 81-100% positive cells. Samples were considered positive when both scores were $\geq 2$. All of the cells identified as trophoblast in each section were evaluated. The sum of the two scores gave a final score between 0 and 8. Statistical analysis of the data was carried out with the Statistical Software Program version 5.0 (Primer of Biostatistics, McGraw-Hill Companies, Inc., New York, NY) using non-parametric Mann-Whitney Rank Sum Test with values considered significantly different when $p < 0.05$.

## Results

Immunohistochemical analysis of normal placental bed and GTDs specimens included identification of the invasive cells as cytokeratin-positive (Figure 1A,C,E) as well as single and double (Figure 1B,D,F) immunostaining for CEA and galectin-3. Semiquantitative scores were derived independently, based on the single staining for either CEA or galectin-3 (Figure 2). Normal placental bed specimens demonstrated weak or absent immunostaining for CEA and galectin-3 in both interstitial and endovascular trophoblast cells. Of the 9 cases of normal placental bed material, 5 were negative for CEA, 4 had both negative and trace positive trophoblast cells (representative staining Figure 1B). Regarding galectin-3, in 4 cases the trophoblast cells were negative and in 5 cases there were both negative and trace positive cells. Semiquantitative assessment of sections gave a mean score for normal interstitial trophoblast (n=9) of $3.5 \pm 0.7$ for CEA and $4.2 \pm 0.7$ for galectin-3 (Figure 2).

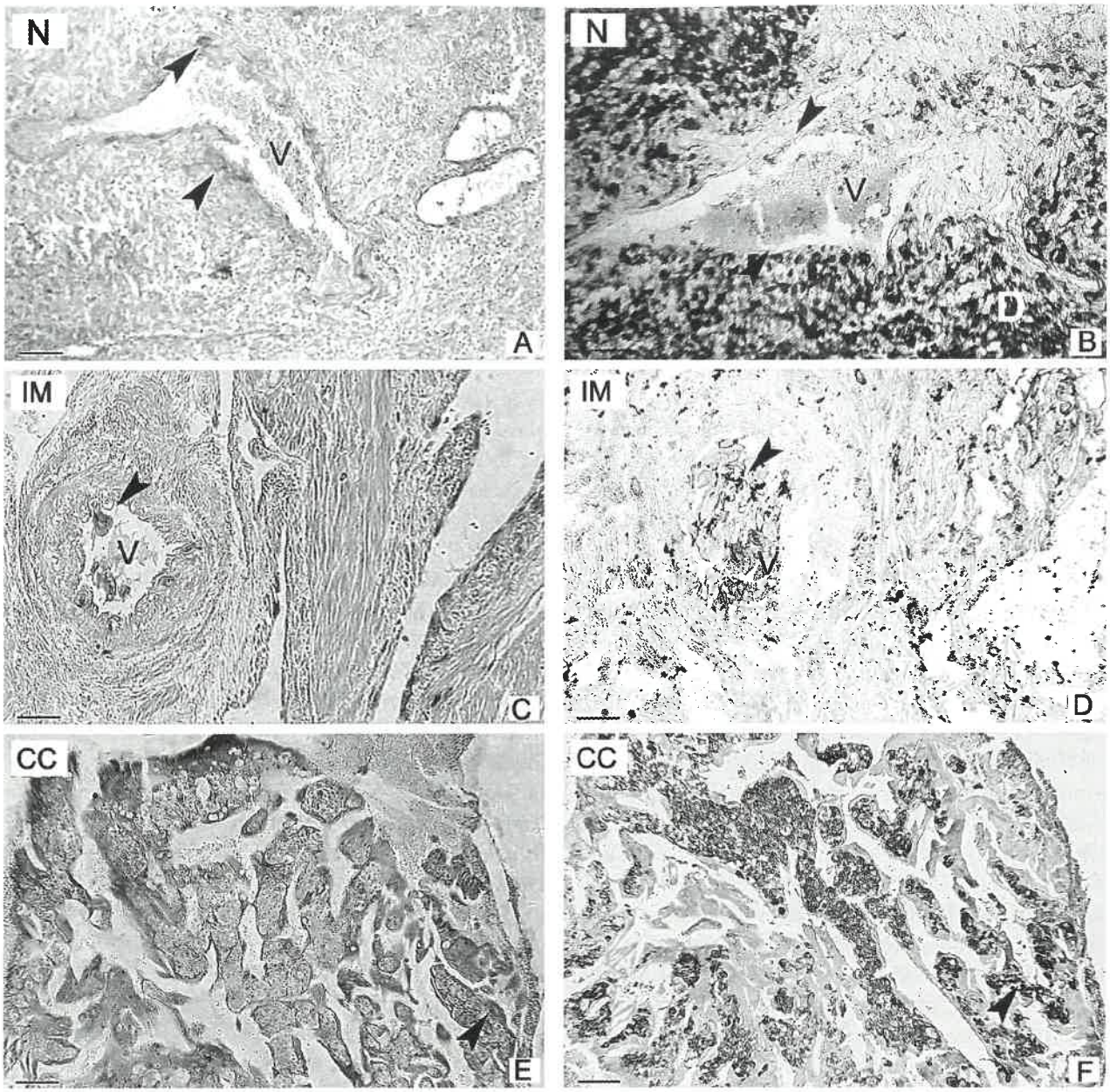



**Figure 1.** Double immunohistochemical staining for CEA and galectin-3 (B,D,F) in cytokeratin identified (A,C,E) invasive trophoblast cells of normal placental bed material (N) (A,B), invasive mole (IM) (C,D) and choriocarcinoma (CC) (E,F). Arrowheads show representative trophoblast cells; D: decidua; V: maternal blood vessel. Scale bar represents 100 $\mu$m.

When the samples of GTDs were examined, CEA was detected in all cases (n=8) of invasive mole. Cytokeratin-positive transformed trophoblast cells (Figure 1C) stained in high percentage for CEA (from 51-100% positive), giving an average score of 5.0 ± 1.1 (Figure 2), which was found significantly different (p á 0.05) from the normal trophoblast. The score for galectin-3 for the same study samples was previously reported [28] to be 6.5 ± 1.1 (Figure 2), which was found significantly different (p á 0.05) from the score for normal trophoblast. The representative photograph for double immunostaining of the invasive mole trophoblast cells is shown in Figure 1D.

In all of the samples of choriocarcinoma, trophoblast stained for both galectin-3 and CEA (Figure 1F) with variable intensity (scores 2-3). The mean score for CEA was 5.8 ± 0.7 (p á 0.05) and 6.1 ± 1.3 (p á 0.05) for galectin-3 (Figure 2).

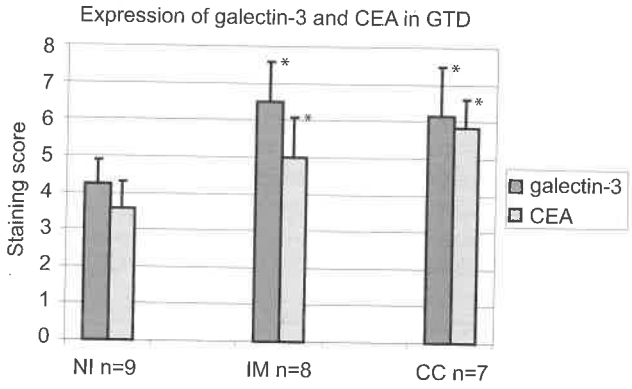



**Figure 2.** Galectin-3 (black column) and carcinoembryonic antigen (CEA) (white column) immunoreactivity in normal placenta (NI), invasive moles (IM) and choriocarcinomas (CC). The immunohistochemically stained specimens were evaluated semiquantitatively. Both the percent of positive trophoblast cells and the staining intensity were taken into account giving final scores. Bars represent the mean ± S.D.; values significantly different when $p < 0.05$ (*).

## Discussion

Both galectin-3 and CEA seem to be implicated in tumorigenesis and metastasis. In addition, some studies have identified CEA as a ligand for galectin-3. The present study was designed to investigate a possible correlation between the expression of galectin-3 and CEA in the transformed trophoblast of the GTDs. A comparison was made to the expression of these molecules in the invasive trophoblast cells of the normal placental bed. An increase in galectin-3 expression in the transformed trophoblast of the invasive mole and choriocarcinoma compared to the control was reported previously [28]. As presented here, CEA was immunolocalized in all specimens of the invasive mole and choriocarcinoma.

The results of this study have shown that the expression of both CEA and galectin-3 is increased in invasive moles and choriocarcinoma compared to the invasive trophoblast of the normal placental bed. This finding may suggest that CEA and galectin-3 are coexpressed in the transformed cells of the studied pathological samples. Their cellular distribution in each case of GTDs was studied by double label immunohistochemistry. The results obtained showed that galectin-3 and CEA were found predominantly in the cytoplasm but were also associated with the cell membrane of the transformed trophoblast. Differences in the relative abundance between the two markers were noted among the immunopositive cells. There were cells that stained strongly for both markers, while other cells stained predominantly for one of the marker.

There is not much data in the literature relating galectin-3 and CEA expression in human tumors. Ohannesian et al. reported binding of galectin-3 to CEA and their co-localization on the cell surface of colon carcinoma cells with CEA [21]. These authors concluded that interactions between galectin-3 and CEA on the cell surface could modulate the distribution of CEA in the membrane and its efficiency as an adhesive molecule. Recently, it was reported that in medullary thyroid carcinoma galectin-3 and CEA are co-expressed in the same tumors at an advanced tumor stage [33,34], indicating the possibility of their autocrine cooperation during tumor progression [33]. Similarly, an increase in galectin-3 and CEA expression at the protein level was recently shown for the solid cell nests of human thyroid gland, manifested as strong and diffuse staining of the cytoplasm for both antigens [35].

At present, it is not clear what are the main functions of galectin-3 and CEA in the GTDs and whether their interaction is in any way responsible for malignant transformation. Increased immunoreactivity for galectin-3 followed by increased immunostaining for CEA in gestational tumors as shown here, could point to CEA as one of the ligands for galectin-3 in transformed trophoblast, which needs to be studied in more detail.

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