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Summary

High dose chemotherapy in breast cancer reviewed. Baars JW, Rodenhuis S, Wall E van der and Schornagel JH. Ned Tijdschr Klin Chem 1995; 20: 288-293.
Laboratory and experimental data show a dose response curve for cytostatic drugs, especially for alkylating agents. For many malignancies, clinical evidence of a dose response relationship is limited .
The dose limiting toxicity of most cytostatic drugs is myelosuppression, which can be circumvented by the use of haematopoietic growth factors and/or autologous bone marrow or peripheral stem cell support.
Clinical data derived from studies in patients with metastatic breast cancer, show that dose escalations of 1.5-2 x standard dosages, possible without autologous bone marrow or peripheral stem cell transport can induce higher remission rates, which did not, however, correspond to a significant survival advantage. Despite promising results from small trials with high dose intensity treatment in combination with peripheral stem cell or bone marrow support (depending on the schedule used, dose escalations possible of 5-10 x the standard dosages) in a selected patient population with high risk or metastatic breast cancer, they do not justify the use of this approach outside the setting of clinical studies. We have to gain more knowledge of selecting the patients who are likely to profit from high dose chemotherapy as well as to continue focusing on improvement of efficacy, reduction of the considerable morbidity and costs of this treatment.
Key-words: breast cancer, high dose chemotherapy, peripheral stem cell transplantation, autologous bone marrow transplantation, haematopoietic growth factors.

Ned Tijdschr Klin Chem 1995; 20: 293-298

Episialin/CA15-3: its structure and involvement in breast cancer progression

J. HILKENS, J. WESSELING, H. L. VOS, J. STORM, M. BOER, S. W. van der VALK and M. C. E. MAAS

Numerous monoclonal antibodies (mAbs) have been raised against mucins on carcinoma cells. Many of these mAbs are directed against an epithelial sialomucin (1, 2, 3, 4, 5) that is now referred to as episialin. Episialin is one of the major sialylated glycoproteins at the surface of most types of carcinoma cells. With a few exceptions, the molecule is only present in normal tissues at the apical side of exocrine glandular cells and is therefore not in direct contact with the circulation. In contrast, on carcinoma cells, the molecule is often expressed in a non-polarized fashion. Episialin is a membrane-bound glycoprotein, but its extracellular domain can be released from the cell and appears in the serum of breast cancer patients. As determined with mAbs that are directed against a non-glycosylated, non-repeat region of the molecule, and by RNA in situ hybridization, the expression of the molecule is strongly increased in carcinoma cells relative to the corresponding normal epithelial cells. For example, we found that the expression level in breast cancer cells is at least 10-fold above the level in normal breast epithelium. The biological background for the up-regulation of episialin expression has not yet been determined.
Several of the mAbs against episialin have been used to develop serum assays. One of these assays is the CA 15-3 test, a sandwich assay using mAb 115D8 developed in our group (6) and mAb DF3 raised by Kufe and colleagues (7). Both mAbs were initially employed in separate assays (the MAM-6 assay (6, 8, 9) and DF3 assay (10)) but have been combined in the CA 15-3 assay where they act as catcher and tracer, respectively.
In this report we will give an outline of the structure of the molecule, discuss the various glycoforms of episialin which explains the variations in results obtained with the various serum assays and review the effect of this elongated molecule on cellular adhesion and metastasis.

Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam

Address correspondence to: Dr. J. Hilkens, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

Structure of episialin

We cloned episialin cDNA and subsequent sequence analysis revealed that episialin is synthesized as a transmembrane molecule with a relatively large extracellular domain and a cytoplasmic domain of 69 amino acids (11). The extracellular domain mainly consists of a region of nearly identical repeats encoding 20 amino acids. The number of repeats is highly variable in the human population, leading to substantial differences in molecular weights of the episialin molecules from different individuals (12). The repeats together with adjacent degenerated repeats contain many serines and threonines which are potential attachment sites for O-linked glycans and constitute the mucin-like domain which comprises more than half of the polypeptide backbone, even in the smallest allele detected. The number of tandem repeat sequences in each allele can vary from approximately 30 to 90.

Figure 1. Models of a cell-bound mucin (episialin) and a secreted mucin. In the model of episialin the protein backbone comprises a tandem array of 40 repeat units (800 amino acids), which is the estimated number of repeats in the short allele of episialin in the T47D breast carcinoma cell line. The extracellular domain of the molecule is almost completely covered by O-linked glycans. Five N-linked glycans are present C-terminal of the repeat region. The variations in the N-terminus due to the alternative splicing are not indicated. A typical transmembrane sequence of at least 24 amino acids is found in the C-terminal part, followed by a cytoplasmic domain of 69 amino acids. The second model shows the macro molecular structure of a secreted mucin as proposed by Carlstedt et al. (1983) and largely confirmed by the deduced aminoacid sequence after cloning several secreted mucin genes. Most of the protein backbone is covered by O-linked carbohydrates, only the N and C-terminal ends contain cysteine rich non-glycosylated domains. These domains are responsible for the multimerization of the individual mucin molecules.

The mucin domain of episialin contains many prolines and other helix-breaking amino acids, resulting in a molecule with an extended structure and many β-turns (13). The extended structure is very rigid as a result of the numerous O-linked glycans attached to the molecule. Consequently, the mucin domain of episialin reaches an extreme length. According to Jentoft (13), an extensively O-linked glycosylated polypeptide of 20 amino acids is approximately 5 nm long. This means that the mucin-like domain of episialin extends 200 to 500 nm above the cell membrane (14). Electron-microscopic analysis of purified episialin molecules as well as episialin present on an in vitro cell line revealed that episialin indeed has the predicted thread-like structure. Figure 1 shows a model of episialin.

Episialin is unlike the genuine mucins that are secreted by specialized cells and form the mucus layer in e.g. the gastrointestinal tract. The genuine mucins are secreted by highly specialized cells such as the goblet cells of the intestine or the mucus producing salivary glands, and form gels covering a large area. Before secretion, they are stored intracellularly in storage vesicles, where they form large oligomers linked by S-S bonds (15). After secretion, the tangled polymers do not remain bound to the cell surface. In contrast, cell surface-bound mucins such as episialin, CD43 and epiglycanin are anchored in the membrane and are not produced by a highly specialized cell type. They are not secreted but shed from the cell surface. These mucins do not form gels. Both types of mucins are depicted schematically in figure 1. The common characteristic of both types of molecules is that a large part of the protein backbone consists of an array of threonine, serine and proline-rich tandem repeats which are heavily O-glycosylated. This region, which in secreted mucins is flanked by a cysteine-rich sequence, constitutes the actual mucin domain. Episialin is one of the best characterized cell surface-associated mucins and may serve as the prototype for this class of molecules.

Biosynthesis of episialin

It has been shown by means of biosynthetic labeling followed by immunoprecipitation that episialin is synthesized as a large single polypeptide, in most cell lines approximately 200 kDa or more (16, 17). This precursor is rapidly cleaved by proteolysis in a smaller moiety, which contains the transmembrane and cytoplasmic domains, and a larger part which comprises most of the extracellular domain. Both moieties remain non-covalently associated (18). This proteolytic processing step occurs in the endoplasmic reticulum and may be essential for further maturation. Episialin is mainly processed by adding numerous O-linked glycans, which increases the apparent molecular weight on SDS-polyacrylamide gels to more than 400 kDa. The extensive glycosylation protects the molecule against proteolytic degradation, since the precursors without O-linked sugars are degraded rapidly, while the mature molecule is extremely resistant to the action of proteases. The glycosylation also determines the rigidity of the molecule as discussed above. The last step in the processing of episialin is the addition of sialic acid to the glycans, which increases the mobility of the molecule on SDS-gels.

The early proteolytic cleavage step is not directly responsible for the release of episialin from the membrane, which suggests that episialin is most likely released from the membrane by a second proteolytic cleavage step after arrival at the cell surface. The second proteolytic cleavage seems to be a slow and probably random process, allowing the mucin to remain associated with the cell surface with a half-life of 16-24 h.

Differential glycosylation of episialin

The repetitive part of the protein backbone of episialin is very immunogenic and most mAbs raised against episialin bind to this part of the molecule, as was shown by their reactivity with synthetic peptides (19), and/or with the repetitive part of the gene expressed in bacterial expression vectors. For example, mAb DF3 strongly reacts with the protein backbone. However, mAb 115D8 reacts with an epitope which involves the protein backbone, but full reactivity is dependent on the presence of carbohydrates, in particular sialic acid. Other mAbs, such as Ca1 (20) and 3E1.2 (21), seem to be directed against epitopes exclusively consisting of carbohydrates. However, it is difficult to prove that no amino acid residues are involved as long as the carbohydrate sequence has not been determined. The peptide sequence of the minimal epitope of some of the mAbs has been deter-

mined. Several epitopes overlap or are only shifted by one or two amino acids (3, 4). Some epitopes include a serine or threonine which are potential glycosylation sites.
Some mAbs directed against peptide sequences, like SM3 (22) and our mAb 175C5, show a high preference for breast carcinomas relative to normal breast epithelium. Other mAbs, like mAb Om-1 raised against episialin on ovarian carcinomas (23) and mAb 202H4, raised in our group against the peptide backbone, react only with specific subsets of epithelial tissues. We investigated the biochemical background of the preferential binding to different tissues of the various mAbs against episialin. For this purpose we used carcinoma cell lines of different tissue origin as models (5). The reactivity of the mAbs is complex. All mAbs directed against the protein backbone precipitate the non-glycosylated precursor molecule from all cell lines expressing episialin. However, the mature molecules are precipitated by some mAbs from only a restricted number of carcinoma cell lines. Even within a single cell line some mAbs precipitate all mature molecules, whereas others precipitate only subsets of the mature episialin molecules. These differences in reactivity are most likely caused by variations in accessibility of the peptide epitopes to the mAbs as a result of differential glycosylation. Thus differentially glycosylated molecules, referred to as glycoforms, each show a restricted expression pattern resulting in positive and negative cells and cell lines for certain mAbs. These results obtained with cell lines can be applied to explain the differential reactivity of certain mAbs with carcinomas and normal epithelial cells and the differential reactivity with tissues of different histological origin.
The difference in O-glycosylation of episialin on tumor cells is also apparent from the direct analysis of the glycans. The most frequently present O-linked glycans on episialin from BT-20 breast carcinoma cells and from HEp 2 larynx carcinoma cells are relatively short (1-4 sugars) and consist of sialic acid, galactose and galactosamine (24, 25). In contrast, the glycans in episialin from human milk, and thus probably also in episialin from breast alveolar epithelial cells, are much longer and more complex (26). In addition to differences in length or branching of the glycans, differential glycosylation could occur by differential initiation of the O-linked glycans.
It is important to realize that in most cases the differential reactivity is not an all-or-nothing phenomenon, but a preferential reactivity with certain tissues or tumors. Even within one type of carcinoma, the number of epitopes accessible for a given anti-episialin mAb may vary in each individual tumor.

Episialin expression and cellular adhesion

To investigate the effect of overexpression of episialin on tumor cells, we transfected several cell lines with episialin cDNA under the control of the CMV immediate early promotor. A proportion of the cells of the transfected cell clones were growing in suspension (figure 2), whereas the adhesion of control cells was not affected, indicating that cell-matrix interactions were reduced. Indeed, adhesion of the transfectants to individual extracellular matrix components such as laminin, fibronectin and collagen I and IV was also strongly affected, confirming the notion that episialin reduces the integrin mediated adhesion (27). Episialin-negative revertant cells were used as controls, which have been bulk-selected with the cell sorter.
In addition to the reduced cell-matrix adhesion, the transfected cells also exhibit a reduced cell-cell adhesion as compared with the revertants as was shown in cell aggregation assays (28). In fact, overexpression of episialin on one of two interacting cells is already sufficient to inhibit aggregation.
As discussed above, episialin is towering 200-500 nm above the plasma membrane, whereas most proteins at the cell surface remain inside the boundaries of the glycocalyx which is approximately 10 nm thick. Therefore, we have tested whether the size of the molecule is responsible for the anti-adhesion effect of episialin. Episialin molecules in which we had

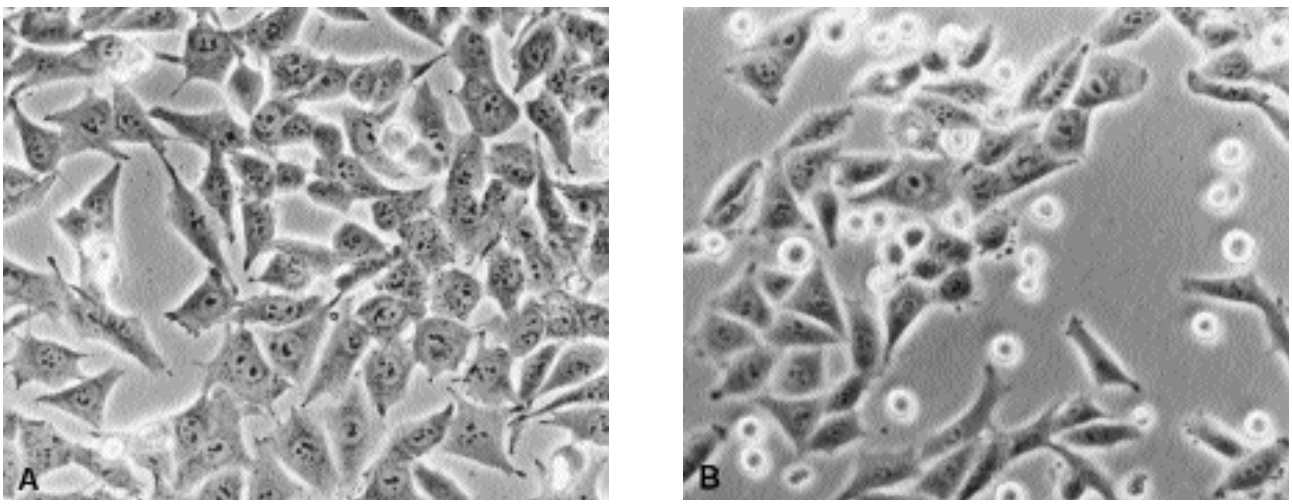

Figure 2. The anti-adhesion effect of episialin. A proportion of the melanoma cells transfected with episialin cDNA does not adhere to the plastic of a culture flask (B), whereas the revertant cells which have lost episialin expression adhere normally under the same conditions (A).

deleted most of the repeat domain, resulting in a molecule <90 nm, had lost its anti-adhesive properties. Removal of the negatively charged sialic acid and sulphate residues affected the anti-adhesion property of episialin only slightly (Wesseling J, van der Valk S and Hilkens J, in preparation). Thus, the anti-adhesion properties of episialin are the result of the unique elongated and rigid structure of the molecule, while charge repulsion only playes a minor role. We conclude that episialin, if present at a sufficiently high density, can prevent cellular adhesion by hindering the binding of the adhesion receptors to their ligands. Such high densities appear to be present on many carcinoma cells *in vivo* (Hilkens J, unpublished). Interestingly, only the binding to ligands bound to a semi-solid phase, such as the plasma membrane or the extracellular matrix, is prevented by episialin, since mAbs in solution can bind to integrins on episialin-transfected cells in an almost undisturbed way, whereas the same antibodies can no longer reach the integrins on the same cells, when absorbed to plastic (27).

Episialin can promote cellular invasion in vitro

By using E-cadherin/episialin double transfectants, we demonstrated that episialin can prevent E-cadherin mediated cell-cell adhesion. Decreased E-cadherin mediated cell-cell interactions are known to promote invasion in vitro (29). Moreover, loss of E-cadherin expression in several types of cancers correlates with poor prognosis of the patient. Decreased E-cadherin expression and episialin overexpression is likely to have the same effect. Indeed, episialin overexpression in SV-40 transformed mammary epithelial cells (HBL-100) promoted invasion into a reconstituted extracellular matrix (matrigel).

Episialin interferes with immune recognition

As discussed above, episialin prevents cell-cell adhesion. Therefore we assumed that the molecule may also prevent conjugate formation between cytotoxic effector cells and their target cells. Indeed, episialin-transfected A375 melanoma cells were unable to efficiently form conjugates with rIL-2 activated large lymphocytes (LAK cells) and allogeneic T lymphocytes (CTL), stimulated with A375 cells, whereas conjugate formation between the lymphocytes and the revertants, which had lost episialin expression, was hardly affected. Subsequently, we measured the lysis of episialin-transfected A375 melanoma cells and episialin-negative revertants by the LAK cells and CTL with time, in a ^{51}Cr release assay. The kinetics of lysis of episialin-negative cells by the LAK cells was comparable to that of K562 cells, the standard target of LAK cells, whereas lysis of the episialin-positive A375 transfectants, was significantly slower (30). However, the maximal percentage of target cells lysed eventually reached comparable levels. Nevertheless, the slower rate of killing episialin-expressing cells might be crucial to the survival of metastasizing cells.

Episialin expression and experimental metastasis

We injected an episialin-transfected A375 clone and revertants of these cells into the tail vein of 21-28 days old Balb/c nu/nu mice. The mice were killed after 5-7 weeks, and the lungs were weighed; counting of the metastases was not possible due to their large number of nodules obtained with the episialin positive cells. The weight of the lungs of the mice injected with the episialin positive A375 cells was significantly higher than that of the mice injected with revertant cells, whereas the growth rate in vitro did not differ between the transfectants and revertants. In some animals injected with revertant cells we observed a relatively high number of metastases. About 25% of these metastases turned out to be positive for episialin, whereas less than 1% of the injected cells were episialin-positive as shown by FACScan analysis, which suggests that episialin-positive cells have a higher propensity to metastasize. Other tissues were only rarely affected (as determined at the macroscopic and microscopic level) with the exception of the brain. We conclude that episialin positive cells metastasize more efficiently to the lung than episialin negative cells. Since episialin protects cells against immune attack of LAK cells (closely related to NK cells that form the main cellular immune defense of nude mice) *in vitro*, it is tempting to speculate that the difference in the formation of experimental metastases is due to escape of the episialin positive cells to immune destruction. Alternatively, the differences in adhesion properties of the episialin transfectants and revertants may cause the difference in metastatic potential between both cell types.

Episialin and progression of breast carcinomas

Cellular adhesion is important for metastasis; in particular cell-cell adhesion as discussed above, but also cell-matrix adhesion has an effect on breast tumor dissemination which is not yet fully understood.

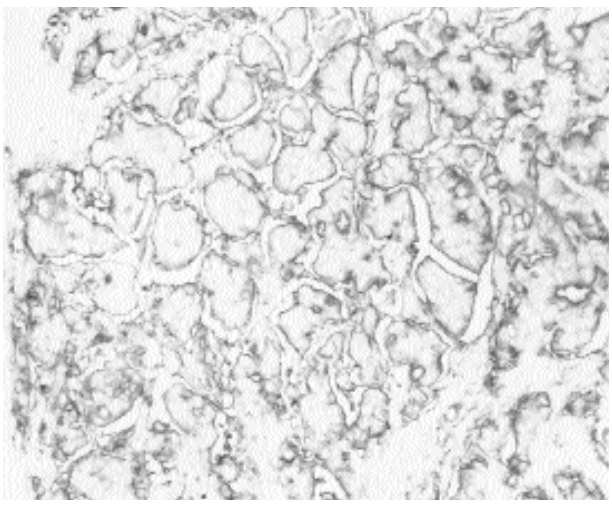

Figure 3. Cleft formation and episialin staining at the tumor-stroma boundary. A ductal breast carcinoma was immunostained with mAb 214D4 directed against episialin. Only limited or no cell-stroma contacts are present in those tumor margins at which episialin staining is present.

Since episialin has an effect on both types of adhesion, we investigated the role of episialin in metastasis. We found that in primary breast cancers there are several patterns of episialin expression of which more than one type can be found in a single tumor. Episialin may be exclusively present at the apical membrane (in highly differentiated tumors), at the entire cell surface (non-polarized), intracellularly, at the entire cell surface and intracellularly, or at the basal site (facing the stroma) of tumor cell clusters. In the latter case mainly those cells lining the stroma were staining for episialin. Cells expressing episialin at the basal site made almost no cell-stroma contacts (figure 3). Thus, the expression of episialin may also affect cellular adhesion *in vivo*. Interestingly, patients having primary ductal breast tumors, exhibiting the latter inverted expression pattern, showed lymph node involvement significantly more often than patients with one or more of the other patterns of episialin staining ($p<0.05$).

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Summary

Episialin/CA15-3: its structure and involvement in breast cancer progression. Hilkens J, Wesseling J, Vos HL, Storm J, Boer M, Valk SW van der and Maas MCE. Ned Tijdschr Klin Chem 1995; 20: 293-298.

Episialin, also designated EMA, PEM, CA 15-3 antigen etc., is a highly polymorphic epithelial sialomucin, which is encoded by the MUC1 gene. Episialin is present at the apical side of glandular epithelial cells. Its mucin-like extracellular domain protrudes high above the cell surface. It is often present at increased levels in breast carcinomas relative to normal breast epithelial cells. In patients with breast and other carcinomas, episialin is released from the cancer cells into the circulation and can be detected by the CA 15-3 assay. The assay is useful for monitoring the course of the disease and for early detection of recurrent breast cancer.

The episialin molecule is heavily O-glycosylated. As a result of differential glycosylation, there are many different glycoforms of episialin. Some glycoforms are preferentially present on carcinoma cells and others are preferentially present on one or a limited number of tissues. Monoclonal antibodies directed against episialin often specifically bind to one or a subset of glycoforms and thus can have a tumor or tissue preference, which explains the differences between the various serum assays that measure episialin.

We have shown that overexpression of episialin prevents cell-cell and cell-extracellular matrix adhesion by shielding the adhesion receptors. As a result, episialin augments the invasive properties of tumor cells *in vitro*, the resistance of the tumor cells to immune destruction, and the formation of experimental lung metastases following intravenous injection of cells that overexpress episialin into nude mice. Preliminary evidence suggests that a certain pattern of episialin expression in primary breast cancers correlates with lymph node involvement.

Key-words: episialin, CA 15-3, mucin, glycoforms, cell adhesion, metastasis.

Ned Tijdschr Klin Chem 1995; 20: 298-300

Application of tumour markers in mammary carcinoma

C.M.G. THOMAS

Of all malignant tumours in women, breast cancer is the first cause of death in the Netherlands. Early diagnosis by means of mammographic screening is one of the few possibilities to increase the prognosis of breast cancer. Despite the limitations of this type of examination, mammographic screening has made it possible to reduce the mortality of this lethal disease by 30% in the group of women aged 50-70 years.

A tumour marker detectable in serum would be helpful to contribute to the early diagnosis, provided the test were sensitive and specific enough to already mirror minimum amounts of one particular malignant tumour at the initial stage of the disease concerned.

Specific serum tumour marker tests would also be valuable at those stages of disease where there is a need for independent information to evaluate the condition of the patient. Tumour marker determinations in serum could also be used as prognostic factors to predict impending relapse following primary treatment, or as a reason for starting adjuvant or palliative therapy. These results could also help to evaluate the efficacy of that therapy. In addition, if serum tumour marker determinations conducted on a regular basis could identify preclinical relapses in bone or organs, this information could be used to initiate new curative therapies. In the case of metastatic breast cancer, serum tumour marker testing during therapy could play a part in assessing treatment effects. It could be an advantage to simply recognize ineffective treatment at an early stage because this would allow a quicker change to possibly more effective therapy. Finally, the simplicity of the assessment itself might comfort the patient.

Department of Obstetrics and Gynaecology and Laboratory of Endocrinology and Reproduction, University Hospital "St-Radboud", Nijmegen

Address correspondence to: Dr C.M.G. Thomas, Department of Obstetrics and Gynaecology, University Hospital Nijmegen "St-Radboud", P.O. Box 9101, 6500 HB Nijmegen, the Netherlands.

Serum tumour markers for mammary carcinoma

The various categories of potentially useful tumour markers for serodiagnostic use in breast cancer comprise Cancer Antigen 15-3 (CA 15-3), Carcinoembryonic Antigen (CEA), and Tissue Polypeptide Antigen (TPA).

CA 15-3 has been identified on the apical side of alveoli and ducts of mammary glands and as a circulating antigen. Distinct epitopes of this high molecular-weight mucine-like glycoprotein of 300-450 kD (also known as polymorphic epithelial sialomucines, episialin) are identified by monoclonal antibodies DF3 (1) and 115D8 (2).

The oncofoetal protein CEA (3) was one of the first tumour markers tested in breast cancer, but has become well-known by its occurrence in carcinomas of the gastrointestinal tract and lung.

Serum elevations of TPA occur in breast, lung, gastrointestinal, urological and gynaecological cancers. Already described in 1957 (4), this oncofoetal antigen is found in the cytoplasm of epithelial cells and has been reported to be related to the cytoplasmic intermediate filaments.