Expression of Different Carbohydrate Tumour Markers and Galectins 1 and 3 in Normal Squamous and Malignant Epithelia of the Upper Aaerodigestive Tract

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Abstract. *Aim: Tumour markers hold a great relevance in the diagnosis and the follow-up treatment of different kinds of human carcinoma. Although head and neck cancer occurs frequently, there is still lack of appropriate tumour markers. Our investigation on the expression of sialyl Lewis A (CA19-9) in laryngeal carcinomas, consists of systematical analysis of oncofetal carbohydrates and of galectins 1 and 3 in different normal and malignant tissues of the aerodigestive tract. Materials and Methods: Paraffin-embedded sections of normal tongue, vocal cord, larynx, pharynx and epiglottis, representing normal control tissue and laryngeal cancer tissue were incubated with monoclonal antibodies against sialyl Lewis A and X (sLeA and X), Lewis Y (LeY), the Thomsen-Friedenreich (TF) antigen and galectin 1 and 3 (Gal-1 and -3). A staining reaction was carried out with ABC-peroxidase and diaminobenzidine (DAB). Tissue of breast cancer was used as a positive control. Mouse IgM, as isotype control antibody, was used as a negative control. Semi quantitative evaluation was carried out double-blinded, by two independent investigators, including a pathologist. Results: Squamous epithelia of all investigated normal tissues of the aerodigestive tract show nearly the same pattern. Most impressive findings are the very weak expression of Gal-1 and the total absence of the TF antigen. Laryngeal cancer reveals high amounts of sLeA, Gal-1 and the TF antigen. Conclusion: On the basis of our findings in normal tissue of the aeradigestive tract, these three markers qualified as potential tumour markers for carcinoma of the aerodigestive tract. In particular, the high expression of TF in cancer tissue and its absence from the normal tissue is promising for its establishment as a new tumour marker in this field.*

Head and neck cancer consists of cancers affecting the cutaneous tissue, the lip, the salivary glands, the sinuses, the oral cavity, the pharynx and the larynx. Squamous cell carcinoma of the larynx is the most common neoplasm of the head and neck (1). It is the sixth most prevalent neoplasm in the world, with approximately 900,000 cases diagnosed worldwide (2). Prognosis had little improved in the past 30 years. In patients who have survived, pain, disfigurement and physical disability from treatment have had an enormous psychosocial impact on their lives (3). The cancer's progression follows a series of steps through increasing grades of dysplasia to malignancy. Traditional prognostic factors, such as the primary tumour site, tumour stage and histological grade, fail to predict the clinical outcome of individual patients with laryngeal cancer (4-7).

Detection and staging of lymph node metastasis from carcinoma of the larynx is a major challenge for the head and neck surgeon. Clinical palpation and imaging techniques are currently not sufficient for revealling neck metastases preoperatively. Despite recent developments in our understanding over cancer-regulating protein expression, despite improvements in surgical and radiation therapies, and the increased use of combined radio-chemotherapy, approximately 50% of the patients experience locoregional recurrence, distant metastasis, or second primary tumours, which represent the most important causes of treatment failure (8-10).

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Specific tumour markers for head and neck cancer are completely lacking. Tumour markers are of particular relevance in diagnosis and in follow-up treatment. Squamous cell carcinoma antigen (SCCAg), carcino-embryonic antigen (CEA), lipid associated sialic acid, SCC marker (TA-4), serum intercellular adhesion molecule-1 (S-ICAM-1), cytokeratin fragment 21-1 (CYFRA-21-1), sialyl Lewis A (CA19-9) and tissue polypeptide specific antigen (TPS) have been examined for their value in detecting head and neck cancer. However, due to their low sensitivity, these markers have not been clinically proved, as being useful in head and neck cancer (11, 12). In order to improve the prediction of patient outcome, attempts have been made to develop new prognostic markers capable of distinguishing patients with a good prognosis from those who are more likely to experience disease relapse (13).

Therefore, the aim of this study was the systematic investigation of different oncofetal carbohydrates and of galectins 1 and 3 (Gal-1 and Gal-3) as tumour markers in normal tissue of various localisations of the upper aerodigestive tract, compared to several specimens of laryngeal cancer.

Materials and Methods

Normal samples (n=4) of tongue, vocal cord, larynx, pharynx and epiglottis were fixed in 4% buffered formaldehyde and embedded in paraffin. Due to regular histopathology examinations, different specimens of laryngeal cancer (n=5) were chosen. Sections of 3 μm were performed.

Immunohistochemistry. After dewaxing twice in xylol for 10 min, each section was rehydrated in descending alcohol concentrations. Endogenous peroxidase was blocked with methanol/H_2O_2 for 30 min at room temperature (RT). Slides were then washed in phosphate-buffered saline (PBS), pH=7.4. Incubation with normal horse-serum for 30 min, at RT, was carried out to reduce nonspecific background staining. Incubation with the monoclonal antibody was carried out according to details presented in Table I.

In order to remove excessive antibodies, slides were washed twice in PBS for 10 min and were then incubated with biotinylated secondary anti-mouse or anti-rat (Vectastain, Vector laboratories, Peterborough, U.K.) antibodies for 30 min at RT. After washing the slides again in PBS, they were incubated with avidin-biotin peroxidase complex (Vectastain-Elite, Vector Laboratories) for 30 min at RT. The slides were visualised with the chromogen diaminobenizidine (DAB) (Dako, Hamburg, Germany) and counterstained with Mayer's hematoxylin. They were then washed in an ascending set of alcohols. Coverslips were placed after transfer to xylene.

Evaluation was carried out double-blinded with the help of a semiquantitative score (IRS), as described previously (14). In brief, the IRS score was calculated as follows: IRS = SI × PP, where SI is the optical staining intensity (graded as 0, no staining; 1, weak staining; 2, moderate staining and 3, strong straining) and PP the percentage of positively stained cells. The PP was estimated by counting approximately 100 cells and was defined as 0, no staining; 1, <10% staining; 2, 11-50% staining; 3, 51-80% staining and 4, >80% staining.

Table I. *Antibodies used for this study.*

Antigen	Clone	Supplier	Conditions
Sialyl Lewis A	KM231	Calbiochem	Overnight at 4°C
Sialyl Lewis X	KM 93	Calbiochem	1h RT
Lewis Y	A70-CK8	Glycotope	1h RT
Thomsen-Friedenreich	Nemod	Glycotope	Overnight at 4°C
Galectin-1	201002	R&D systems	Overnight at 4°C
Galectin-3	9C4	Novocastra	Overnight at 4°C

RT: Room temperature.

Controls. Sections of mammary carcinoma were used as positive controls. A negative control was performed by replacing the primary antibody by isotype control mouse IgM or by rat IgG, at the same concentration as the primary antibody.

Results

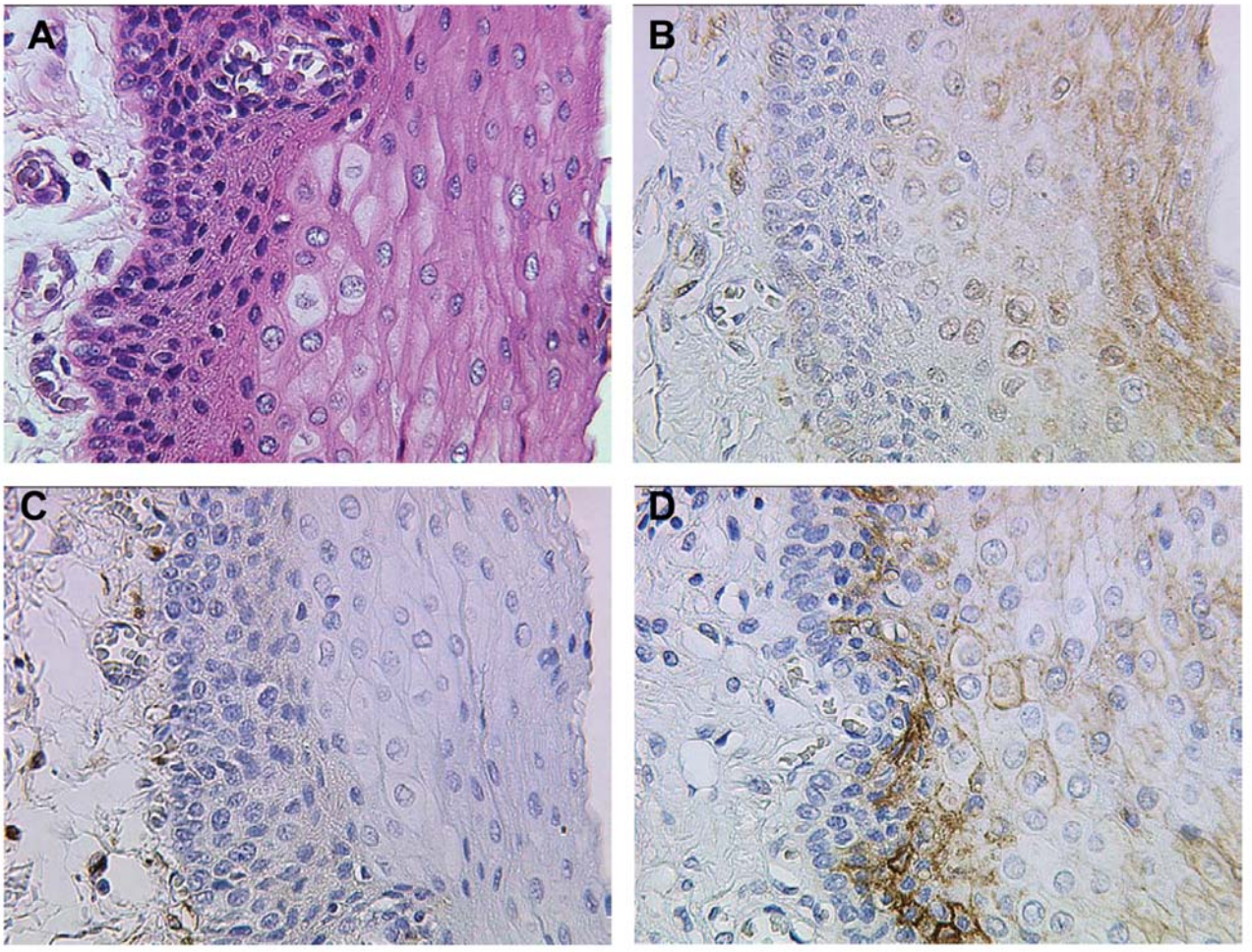
Normal tissue. The squamous epithelial layer of the normal human epiglottis, stained with HE is presented in Figure 1A. Lewis antigens sLeA and sLeX, LeY and Gal-3 presented a moderate expression, on average, in the normal squamous epithelial layer of tongue, the vocal cord, the larynx, the pharynx and the epiglottis. Gal-1 was only expressed in normal tongue and the vocal cord, but was totally absent from the other tissues. The TF antigen was not detected at all (Figure 1B-D). A summary of the staining results is presented in Figure 1E.

Laryngeal carcinoma. Comparing these findings to the analyses of the malignant squamous epithelia of laryngeal cancer we can only point out sLeA, among the carbohydrate antigens, as having a certain potential for becoming a tumour marker for head and neck cancer. Even though it is stained in a moderate way in normal tissue, its expression increases appreciably in squamous cell cancer (Figure 2A-E).

Carbohydrate antigens in normal versus malignant squamous epithelia. When relating the results for the TF antigen and Gal-1 in normal and malignant squamous epithelia of the upper aerodigestive tract, we came across impressive findings. The TF antigen is totally absent from all tested normal tissues and Gal-1 has a low IRS score only in the tongue and the vocal cord, but has no staining in the other tissues. In squamous epithelia of laryngeal cancer, there was a dramatic up-regulation of both markers (Figure 3).

Discussion

The results of this pilot study provide a good indication as for where to focus the search for adequate tumour markers in head and neck cancer. The results for the TF antigen and Gal-1 expression in malignant squamous epithelia of the



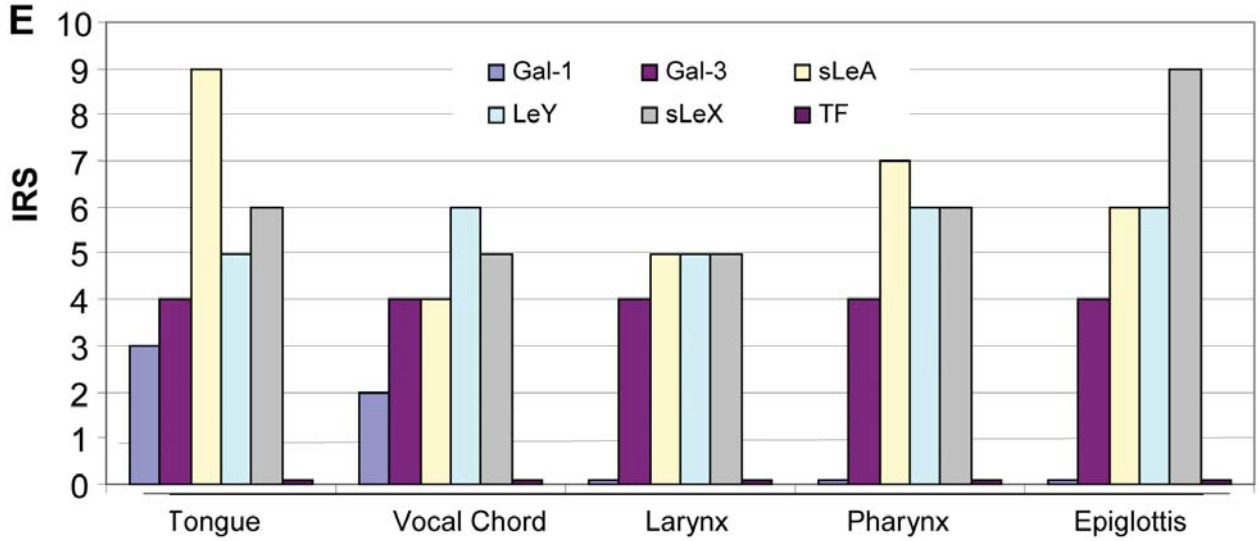


Figure 1. *Squamous epithelial layer in normal epiglottis. A: Mayer's hematoxylin (HE), B: sialyl Lewis A (sLeA), C: galectin 1 (Gal-1), D: galectin 3 (Gal-3), all magnifications 40× lens; E: summary of staining results in normal tissue.*

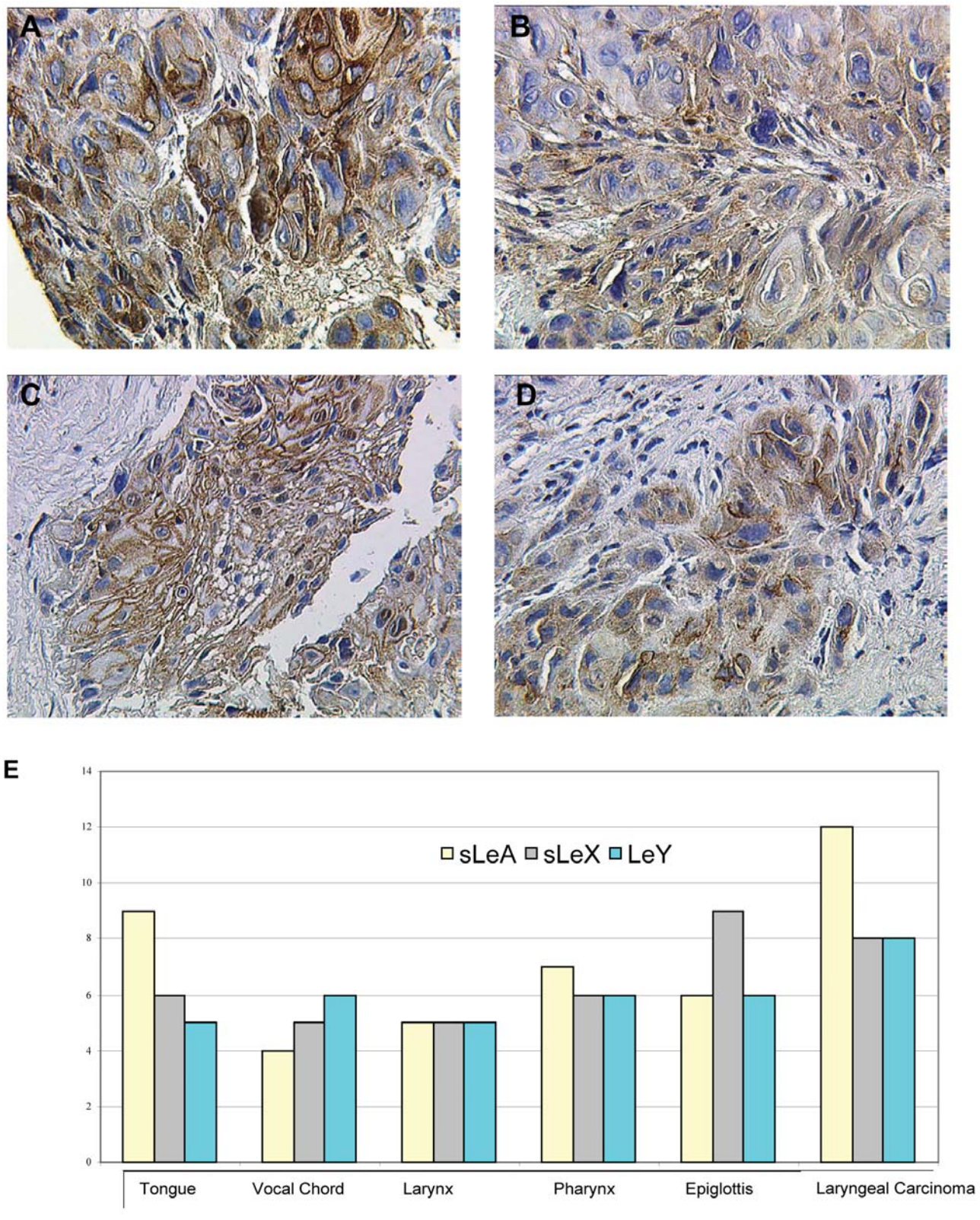


Figure 2. *Squamous epithelial layer in laryngeal carcinoma. A: Sialyl Lewis A (sLeA), B: galectin 1 (Gal-1), C: galectin 3 (Gal-3), D: Thomsen-Friedenreich antigen (TF), all magnifications 40× lens; E: Summary of staining results in carcinoma tissue.*

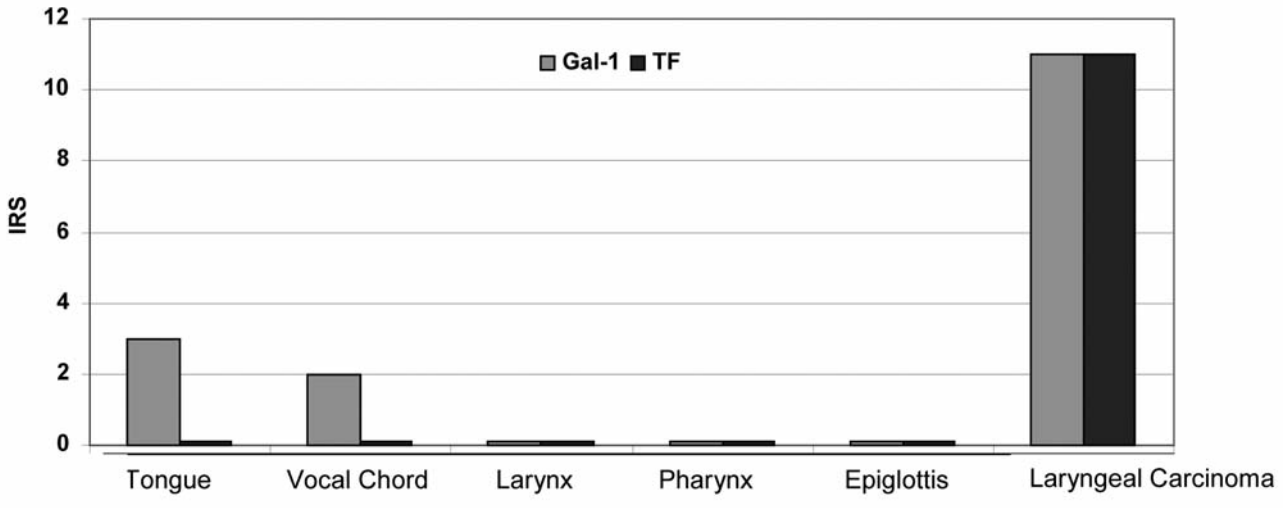


Figure 3. *Thomsen-Friedenreich antigen (TF) and galectin 3 (Gal-3) expression summarized in normal versus malignant squamous epithelia.*

upper aerodigestive tract compared to normal control tissue showed the most promise.

The TF epitope is strongly associated with cancer and was expressed only in a few normal tissues, such as in different organs of human reproduction. TF is a carbohydrate moiety related to blood group epitopes and consists of galactose-β1-3N-acetylgalactosamine (Galβ1-3GalNAc-). In epithelial cells, the TF is carried by mucin-1 (MUC1) on the apical surface of these cells. On tumour cells MUC1 is post-translationally modified resulting in incomplete O-glycosylation and exposure of TF (15). In addition, in the first trimester of pregnancy, we found previous studies showing a strong expression of the TF epitope and of MUC1 at the apical side of the syncytiotrophoblast directed towards the maternal blood. This expression was consistent in the second trimester of pregnancy, and to a lesser degree in the third trimester (16). We also identified positive staining for the TF epitope and for MUC1 on extravillous trophoblast cells in the decidua during the first and second trimester of pregnancy. Trophoblast tumour cells of the cell line BeWo, which form a syncytium *in vitro*, were also positive for the TF epitope and for MUC1, whereas Jeg3 cells, which are unable to form a syncytium, expressed only MUC1 (17). In addition, in several tumour entities, such as gastric (18), colony (19), lung cancer (20), and in cancer of the *cervix uteri* (21), a correlation between TF expression and negative prognosis was demonstrated. Its prognostic impact in other tumour locations, especially in breast cancer, was also investigated. A correlation between high tumour stage and TF expression was observed (21), although in another study, high TF expression predicted improved survival (22). In former studies, we demonstrated that TF is also expressed on disseminated tumour cells in the bone marrow of breast cancer patients (23). In another study, patients with TF-positive breast tumours had a favourable prognosis. We hypothesised that the immunogenicity of TF could lead to an immune response and therefore to a reduction of TF-positive tumour cells in patients with breast cancer (24).

Gal-1, a proto-type galectin, forms non-covalently associated homodimers under physiological conditions with two carbohydrate recognition domains, which preferentially recognise type I and type II *N*-acetyllactosamine residues present on all complex *N*-linked and many O-linked glycoproteins (25, 26). By recognizing cell surface β-galactosidic residues, the lectin displays a wide range of biological activities involving cell adhesion to the endothelium (27, 28). A recent study showed that Gal-1 expression increased in the late secretory-phase of the endometrium and in the decidua (29). In a former study we showed that Gal-1 recognises appropriate glycoepitopes on the syncytiotrophoblast and on chorionic carcinoma cells (BeWo) (30). Results further demonstrate, that the ligation of Gal-1 to Galbeta1-4GlcNAc and to Galbeta1-3GalNAc (TF) epitopes on BeWo cells have regulatory effects on human corionic gonadotropin (hCG) and on progesterone production. In addition we performed binding experiments of irregularly fertilized oocytes using human uterine epithelial Ishikawa cells as a model for TF expressing glandular epithelial cells of the endometrium. Ishikawa cells represent an optimised model of the human endometrium (31). It is a human uterine epithelial cell line that has maintained polar organisation to a high degree (32, 33). In addition Camby *et al*. demonstrated Gal-1 expression as being related to tumour progression and they believed, it

must also be considered regarding dissemination of tumour cells into surrounding normal tissues and regarding tumour immune escape (34). These findings were confirmed by a study of Rabinovich, in which the author emphasized on galectins as potential targets of anticancer drugs (35). In a recent study, we investigated endometrioid adenocarcinomas and found high binding of Gal-1 to stage III/IV tumours and an association with lymphangiosis (36). An additional study determined the highest Gal-1 expression, found in fibroid tissues and similar expression patterns were found in the human myometrium and in leiomyosarcomas (37).

In summary, most impressive findings are the very weak expression of Gal-1 and the total absence of the TF antigen in normal tissue of the head and neck region. Laryngeal cancer reveals high amounts of sLeA, Gal-1 and of the TF antigen. In particular, the high expression of TF in cancer tissue and its absence from normal tissue is promising for the establishment of a new tumour marker in this field.

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