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# The Thomsen–Friedenreich (T) Simple Mucin-type Carbohydrate Antigen in Salivary Gland Carcinomas

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**The simple mucin-type T (Thomsen–Friedenreich) antigen is a marker of carcinomas, and has been related to aggressiveness of malignant tumours. We studied the expression of T, sialosyl-T, A and H blood group antigens in salivary gland carcinomas. The aim was to study whether the tumours, based on the expression of these structures, could be divided into new diagnostic groups that may later show prognostic significance.**

**Formalin fixed paraffin-embedded tissue sections from 77 salivary gland carcinomas of different histological types were studied using immunohistology and monoclonal antibodies (MAbs). Fresh frozen tissue was examined in 30 of the cases. Frozen sections were superior to paraffin sections in demonstrating T and H antigens.**

**Aberrant glycosylation with accumulation of T (in cytoplasm) and sialosyl-T antigens (in cytoplasm, membrane and mucin) was found in all tumour types except acinic cell carcinomas. In carcinomas in pleomorphic adenomas (CinPA) the effect of fixation was minimal and T antigen location was different. In carcinomas with myoepithelial cell (MEC) participation, the MECs had retained a normal glycosylation pattern. H antigen was expressed in all tumour types, except acinic cell carcinomas and CinPA. A antigen was expressed in all tumour types from blood group A patients, except in CinPA. The expression of T, sialosyl-T, H and A antigens in relation to differentiation grade varied with tumour type in poorly differentiated areas. High and moderate differentiated areas were always stained, whereas poorly differentiated areas in some tumour types expressed T and sialosyl-T antigens and others did not. The accumulation versus lack of expression of the investigated structures in poorly differentiated areas of the tumours may be of prognostic significance.**

**Keywords: salivary gland carcinomas, simple mucin-type carbohydrate antigens, Thomsen–Friedenreich antigen, T antigen**

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## INTRODUCTION

SALIVARY GLAND carcinomas are rare tumours which may arise in major and minor salivary glands. The prognosis is dependent on clinical stage, anatomical location, tumour size, histological type and grade of the tumours and radicality of operation [1]. The treatment of patients with salivary gland carcinomas is difficult due to the rarity, as well as histological heterogeneity, of these tumours [1, 2]. To improve treatment, it would be of interest to find markers which were able to recognise tumours with a more aggressive course in order to optimise treatment and follow-up. In this context, simple mucin-type carbohydrate antigens T (Thomsen–Friedenreich), Tn and sialosyl-Tn antigens have gained considerable interest as they accumulate in some carcinomas, and have been related to the clinical course of the tumours (see [3] for an overview; [4–6]). In normal tissues and secretions these structures are cryptic due to further glycosylation and/or sialylation, which may lead to the formation of simple mucin-type (type 3 chain) ABH antigens or to complex carbohydrate structures [7–9]. Few studies have described the distribution of simple mucin-type carbohydrate antigens in salivary gland carcinomas [10–15]. We found recent accumulation of Tn and sialosyl-Tn in salivary gland carcinomas with a glandular differentiation pattern (e.g. adenocarcinomas, mucoepidermoid carcinomas and (adeno)carcinoma in pleomorphic adenoma), whereas adenoid cystic carcinoma and acinic cell carcinoma only expressed minimal amounts of these structures [16]. In the present study we describe the expression of T (Thomsen–Friedenreich) antigen and A and H blood group antigens, which are elongated variants thereof, in relation to histopathological typing and grading of salivary gland carcinomas, in order to investigate whether the tumours,

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based on the expression of the investigated carbohydrate structures, could be divided into new diagnostic groups that may later show prognostic significance.

## MATERIALS AND METHODS

*Patients and tissues*

Tissues from 77 patients who had undergone surgery for different types of carcinoma of the salivary glands were studied. The tumours were reclassified according to the WHO classification [2]. The histological type and anatomical location of the tumours are seen in Table 1. With regard to adenoid cystic carcinoma, 18 primary tumours and four recurrences were investigated. In one case, the primary tumour, as well as the local recurrence, was studied. From each tumour, a tissue block was chosen that usually included adjacent normal salivary gland tissue. Since simple mucin-type carbohydrate antigens are blood group related antigens, the ABO(H) blood group of the patients was obtained by routine procedures (Table 2). Tissue blocks from all patients had been fixed routinely in 10% buffered neutral formalin at room temperature and embedded in paraffin by routine histological procedures. In order to evaluate the influence of fixation and embedding on the antigen preservation we examined freshly frozen tissue specimens from 30 of the tumours (two mucoepidermoid carcinomas, 11 adenocarcinomas, three polymorphous low grade adenocarcinomas, two carcinomas in pleomorphic adenoma, nine adenoid cystic carcinomas, and three acinic cell carcinomas). These specimens had been quick frozen in liquid isopentane precooled on dry ice, and stored at −80°C.

*Antibodies*

Mouse monoclonal antibodies with well-defined specificity for simple mucin-type T, H and A antigens were used. The antibodies, their isotype, and reference for the procedure for their generation, isolation and specificity are shown in Table 3.

All antibodies were hybridoma culture supernatants containing approximately 10–30 μg immunoglobulin/ml, except Mbr1 which was used as ascites fluid (diluted 1:300).

*Immunohistology*

Four micrometre thick sections were cut from paraffin or frozen tissue blocks. Paraffin sections were deparaffinised and rehydrated according to routine procedure, while frozen sections were dried for 2 h and thereafter fixed in acetone for 10 min at room temperature. All sections were mounted on gelatin-coated slides and stained by an indirect immunoalkaline phosphatase technique. Sections were incubated in a moist chamber with MAbs overnight at 4°C, after preincubation for 20 min with normal rabbit serum (diluted 1:5, DAKO X902) at room temperature. All MAbs were used undiluted, except MBr1 which was diluted 1:300. Sections were then washed twice in Tris-buffered saline pH 7.6 (TBS), followed by incubation with rabbit–anti mouse conjugated alkaline phosphatase immunoglobulin (diluted 1:50, DAKO D314) for 30 min at room temperature. Sections were washed again for 5 min with TBS pH 7.6 and incubated with a freshly prepared solution of alkaline phosphatase substrate (2.4 mg levamizole (Sigma L9756), 2 mg naphthol-AS-MX-phosphate (Sigma N4875) and 10 mg Fast Red TR salt (Sigma F-1500) in 10 ml 0.1 M Tris buffer, pH 8.2 for 30 min at room temperature; washed in distilled water and finally counterstained with haematoxylin and mounted with Glycergel (DAKO). Additional paraffin sections of tissue were stained with MAbs HH8 and Hb-T after preincubation for 2.5 h at 37°C with Neuraminidase from *Clostridium perfringens* type X (Sigma),

*Table 1. Anatomical location and histological types of 77 patients with salivary gland carcinomas*

| | Location | | | | |
|---|---|---|---|---|---|
| Histological type | Parotid gland | Submandibular gland | Sublingual gland | Minor salivary glands | Total |
| Mucoepidermoid carcinoma | 11 | 0 | 0 | 4 | 15 |
| Adenocarcinoma | 9 | 3 | 1 | 6 | 19 |
| Polymorphous low grade adenocarcinoma | 0 | 0 | 0 | 3 | 3 |
| Carcinoma in pleomorphic adenoma | 9 | 0 | 0 | 0 | 9 |
| Adenoid cystic carcinoma | 5 | 3 | 2 | 12 | 22 |
| Acinic cell carcinoma | 9 | 0 | 0 | 0 | 9 |
| Total | 43 | 6 | 3 | 25 | 77 |

*Table 2. Histological type in relation to ABO bloodgroup of 77 patients with salivary gland carcinomas*

| | Bloodgroup | | | | | |
|---|---|---|---|---|---|---|
| Histological type | O | A | B | AB | Unknown | Total |
| Mucoepidermoid carcinoma | 9 | 2 | 3 | 0 | 1 | 15 |
| Adenocarcinoma | 4 | 11 | 2 | 0 | 2 | 19 |
| Polymorphous low grade adenocarcinoma | 1 | 2 | 0 | 0 | 0 | 3 |
| Carcinoma in pleomorphic adenoma | 0 | 7 | 1 | 0 | 1 | 9 |
| Adenoid cystic carcinoma | 3 | 19 | 0 | 0 | 0 | 22 |
| Acinic cell carcinoma | 3 | 5 | 1 | 0 | 0 | 9 |
| Total | 20 | 46 | 7 | 0 | 4 | 77 |

*Table 3. The antigens, their structure, the mouse monoclonal antibodies, their isotype and references for their generation*

| Antigen | Antigenic structure | Antibody/ isotype | Reference/Source |
|---|---|---|---|
| T | Galβ1 – 3GalNAcα – R | HH8/IgM<br>HB-T/IgM | [17]<br>DAKO |
| H | Galβ1 – 3GalNAcα/β – R<br>2<br>Fucα1 | MBr1/IgM<br>HH14/IgM | [18, 19]<br>Clausen and Hakomori (unpublished data) |
| A | GalNAcα1 – 3Galβ1 – 3GalNacα/β – R<br>2<br>Fucα1 | HH5/IgM | [20] |
| A repetitive | GalNAcα1 – 3Galβ1 – 3GalNAcα1 –<br>2<br>Fucα1<br>– 3Galβ1 – 4GlcNAcβ1 – R<br>2<br>Fucα1 | TH1/IgG2A | [21] |

0.1 Unit per ml in 0.1 M acetate and 0.04 M $CaCl_2$ buffer, pH 5.5 (frozen sections were preincubated for 30 min.) This treatment was performed to determine possible masking of the antigen with sialic acid. For control of the staining reaction, the primary antibody was replaced by (1) TBS; (2) monoclonal antibodies of other specificities but with the same immunoglobulin isotype as the test antibody; (3) supernatant from the myeloma cell line, Sp2, used for hybridisation for generation of the antibodies.

*Evaluation*

Sections with more than 5% stained cells were recorded as positive; the location to the cytoplasm (related to the supranuclear area, suggesting a location to the Golgi area or homogenously in the entire cytoplasm), at luminal membranes and/or mucinous contents was registered.

## RESULTS

The staining results of the tumours are seen in Table 4 and illustrated in Figs 1–4. The study showed that frozen tissue sections were superior to formalin fixed paraffin-embedded tissue sections in demonstrating T and H antigens, whereas demonstration of sialosyl-T and A antigens did not vary with fixation. The expression of T, sialosyl-T and H antigens was independent of ABO(H) blood group, whereas mucin-type A antigen was only expressed in blood group A specimens. Thus, no incompatible A antigen was found in the tumours (i.e. expression of A antigen in tumours from blood group B or O patients).

*Salivary gland tissue adjacent to carcinomas*

Salivary gland tissue adjacent to tumours showed a staining pattern similar to that seen in normal salivary glands [22, 23]. The staining pattern was identical in similar cell types in major salivary glands, whereas minor salivary glands differed slightly from major salivary glands in serous acinar and duct cells. In brief, mucin-type A antigen was expressed in the supranuclear area of serous and mucinous acinus and duct cells, and in the luminal membrane of ducts in major salivary glands. Serous acinus cells and duct cells in minor salivary glands never expressed A antigen. H antigen was located to the supranuclear area of mucous acinus cells, but to the entire cytoplasm of serous acinus cells and duct cells. T and sialosyl-T antigens were expressed in cells located at the abluminal aspect of acinus and duct cells (i.e. myoepithelial cells and basal cells), and occasionally in duct cells. The expression of T and sialosyl-T antigens in duct cells was predominantly seen in minor salivary glands.

*Salivary gland carcinomas*

The different types of salivary gland carcinomas displayed a staining pattern which differed from normal salivary gland tissue and was independent of anatomical location. Primary tumours stained similarly to local recurrences. The number of stained cells vaired from staining a few to almost all the cells in the tumours.

Based on the expression of T and sialosyl-T antigens, the tumours could be divided into two groups: tumours which showed accumulation of T and sialosyl-T antigens (i.e. mucoepidermoid carcinomas, adenocarcinomas adenoid cystic carcinomas, polymorphous low grade adenocarcinomas and carcinomas in pleomorphic adenoma) and tumours which never expressed T and sialosyl-T antigens (i.e. acinic cell carcinomas). The T antigen positive tumours were stained in the cytoplasm of tumour cells. Sialosyl-T antigen positive tumours were also stained at the membrane and in the mucinous contents of glandular differentiated structures. Furthermore, sialosyl-T antigen was expressed in more cells in individual tumours than T antigen. In T and sialosyl-T antigen positive tumours staining was always seen in the more differentiated areas. In contrast, staining of poorly differentiated areas varied with the tumour type (i.e. adenocarcinomas were stained, but adenoid cystic carcinomas and carcinomas in pleomorphic adenomas were not stained). Within the group of T antigen positive tumours, carcinomas in pleomorphic adenomas differed from the other tumours because: (1) T antigen was expressed in the cytoplasm as well as at the membrane and in the mucinous contents of glandular differentiated structures and (2) expression of T antigen did not vary with fixation.

*Table 4. Distribution of T, sialosyl-T, H and A simple mucin-type carbohydrate antigens in 77 salivary gland carcinomas*

| Histological type | No. of tumours | | Antigenic structures | | | |
|---|---|---|---|---|---|---|
| | Paraffin | Frozen | T | Sialosyl-T | H | A |
| Mucoepidermoid carcinoma | 15 | 2 | PT: 4/15; C, (ME)<br>FT: 2/2; C | PT: 13/15; C, LM, MU<br>FT: 2/2; C, LM, MU | PT: 0/15<br>FT: 1/2; C | PT=FT: 3/3*; $C^s$ |
| Adenocarcinoma | 19 | 11 | PT: 0<br>FT: 10/11; C, (1/11; ME, MU) | PT: 12/19; C, LM, MU<br>FT: 10/11; C, LM, MU | PT: 0/19<br>FT: 7/11; C | PT=FT: 4/10*; $C^s$ |
| Carcinoma in pleomorphic adenoma | 9 | 2 | PT: 7/9; C, LM, MU<br>FT: 1/2; C, LM, MU | PT: 8/9; C, LM, MU<br>FT: 1/2; C, LM, MU | PT: 0/1<br>FT: 0/2 | PT=FT: 0 |
| Adenoid cystic carcinoma | 22 | 9 | PT: 0/22<br>FT: 9/9; C | PT: 12/22; (C), LM, MU<br>FT: 9/9; C, LM, MU | PT: 0/22<br>FT: 3/9; C, (LM) | PT=FT: 14/19*; $C^s$, C, LM, MU |
| Polymorphous low grade adenocarcinoma | 3 | 3 | PT: 1/3; C, ME<br>FT: 3/3; C | PT: 2/3; C, LM, MU<br>FT: 3/3; C, LM, MU | PT: 0/3<br>FT: 2/3; C | PT=FT: 2/2; $C^s$, C, LM, MU |
| Acinic cell carcinoma | 9 | 3 | PT: 0/9<br>FT: 0/3 | PT: 0/9<br>FT: 0/3 | PT: 0/9<br>FT: 0/3 | PT=FT: 2/4*; $C^s$ |

PT, paraffin-embedded tumours; FT, frozen tumours; C, cytoplasm homogenously, $C^s$, cytoplasm, supranuclear area; LM, luminal membrane; MU, mucin. *Blood group A individuals.


**Fig. 1. Mucoepidermoid carcinoma, alkaline phosphatase × 80. (a) T antigen in the cytoplasm and membrane of squamous cells (arrow) (frozen section). (b) Sialosyl T antigen in the cytoplasm and membrane of squamous cells (arrow), intermediate cells (arrow) and mucin of glandular structures (arrowhead) (frozen section). (c) Simple mucin-type A antigen in the supranuclear area of mucous cells and intermediate cells (arrowhead) (paraffin section).**

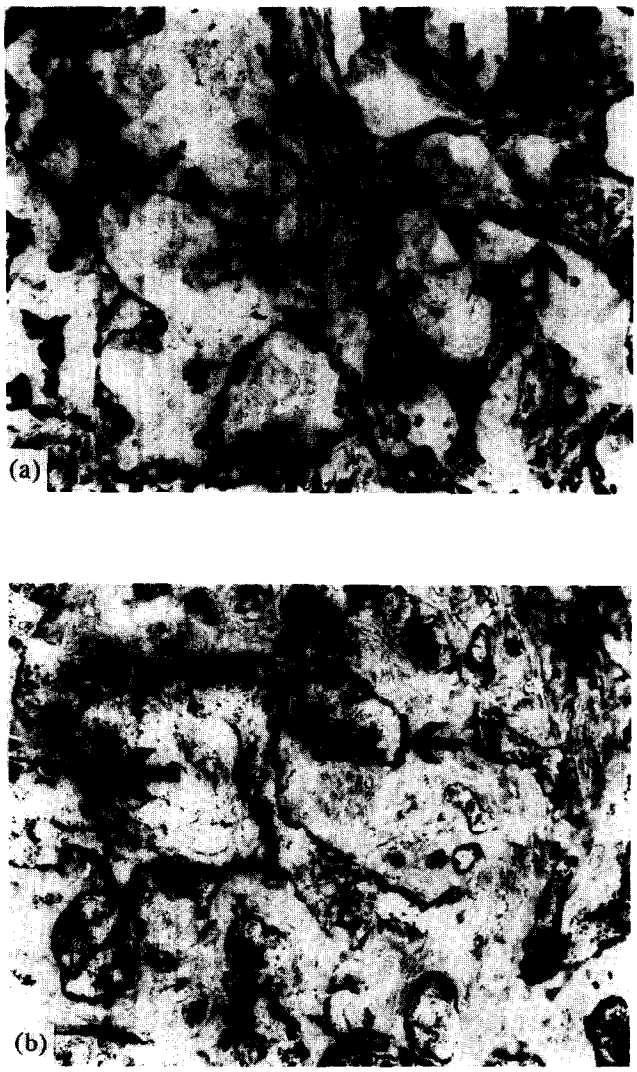


**Fig. 2. Adenocarcinoma, alkaline phosphatase × 80. (a) T antigen in the cytoplasm of tumour cells in relation to glandular structures (arrow) (frozen section). (b) Sialosyl-T antigen in the cytoplasm, luminal membrane (small arrow) and mucin of glandular differentiated structures (large arrow) (frozen section). (c) Simple mucin type A antigen in the supranuclear (Golgi) area of cells (arrowhead) (paraffin section).**


**Fig. 3. Carcinoma in pleomorphic adenoma, alkaline phosphatase × 80. (a) T antigen in the cytoplasm, cellular membrane and mucin of glandular structures (paraffin section). (b) Sialosyl-T antigen in the cytoplasm, cellular membrane and mucin of glandular structures (paraffin section).**

Based on the participation of different cell types in the tumours and the expression of all the investigated carbohydrate structures, tumours could be divided into two groups: (1) tumours with the participation of myoepithelial cells (i.e. adenoid cystic carcinomas and polymorphous low grade adenocarcinomas) and (2) tumours without the participation of myoepithelial cells. The myoepithelial cells in the tumours stained identically to normal myoepithelial cells. These cells had therefore retained a normal glycosylation pattern.

Based on the expression of H antigens, the tumours could be divided into two groups: (1) tumours which had retained the ability to express H antigen (i.e. mucoepidermoid carcinomas, adenocarcinomas, polymorphous low grade adenocarcinomas and some adenoid cystic carcinomas) and (2) tumours which had lost the ability to express H antigen (i.e. acinic cell carcinomas and carcinomas in pleomorphic adenoma). The entire cytoplasm was stained in cells in the well differentiated areas of H antigen positive tumours, whereas cells in the poorly differentiated areas of the tumours were not stained.

Based on the expression of A antigen, the tumours could also be divided into two groups: (1) tumours which had retained the ability to express A antigen (i.e. mucoepidermoid carcinomas, adenocarcinomas, adenoid cystic carcinomas, polymor-

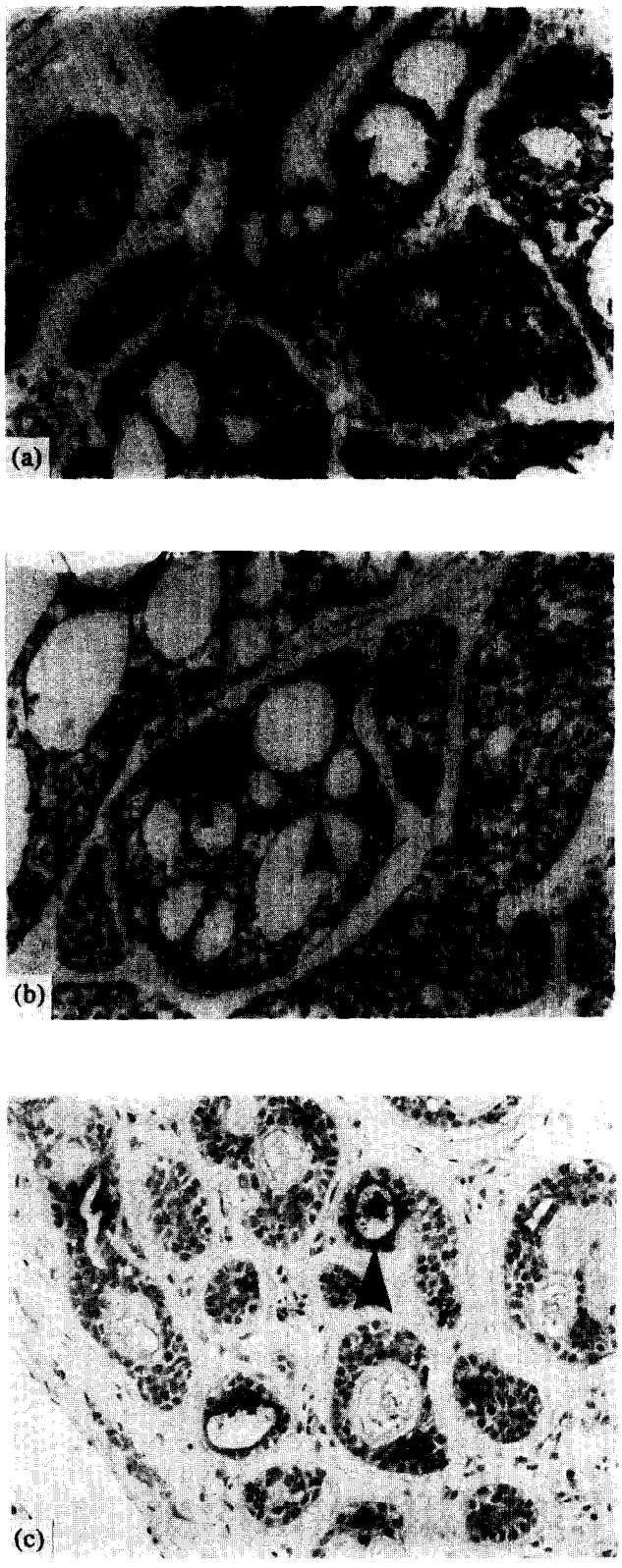


**Fig. 4. Adenoid cystic carcinoma, alkaline phosphatase × 80. (a) T antigen in the cytoplasm of ductular structures (arrow), some myoepithelial cells were also stained (arrowhead) (frozen section). (b) Sialosyl T antigen in the cytoplasm of ductular structures in cribriform area (arrow), more myoepithelial cells were stained when sections had been pretreated with neuraminidase (arrowhead) (frozen section). (c) Simple mucin-type A antigen in the cytoplasm, luminal membrane and mucin of ductular structures in cribriform area (arrowhead) (paraffin section).**

phous low grade adenocarcinomas and acinic cell carcinomas) and (2) tumours which had lost the ability to express A antigen (i.e. carcinomas in pleomorphic adenoma). The A antigen positive tumours were stained in the cytoplasm (supranuclear or entire) and/or the luminal membrane and/or in the mucinous contents of glandular differentiated structures.

## DISCUSSION

We investigated the simple mucin-type T carbohydrate antigen, sialosyl-T and H and A blood group variants thereof in salivary gland carcinomas using immunohistology on frozen and formalin fixed paraffin-embedded tissue sections with a panel of well-characterised MAbs. The demonstration of T antigen vaired profoundly with fixation and embedding, in that all tumours, except carcinoma in pleomorphic adenoma, showed less staining in formalin fixed paraffin-embedded sections than in frozen tumour sections. This influence on fixation and embedding seems to vary with the tissue examined, because no such difference has been found in gastric tissue [24].

The aim of the present study was to investigate whether salivary gland carcinomas, based on the expression of the carbohydrate antigens, could be divided into groups, which may later show prognostic significance. All the different tumour types, except acinic cell carcinomas, showed accumulation of T and sialosyl-T antigens (in the cytoplasm, and in cytoplasm, cellular membranes and mucin, respectively). These findings are in agreement with previous studies in which the lectin PNA (Arachis Hypogeae), that have a broader specificity than MAbs to T antigen [3], was used [12–15].

Carcinomas in pleomorphic adenoma differed from the other tumour types with regard to cytolocation of T antigen. In carcinomas in pleomorphic adenoma, T antigen was expressed in seven of nine tumours with a location to both the cytoplasm, luminal membrane and mucin of the ductular structures, and the only effect of neuraminidase pretreatment was staining of more ductular structures in the tumours and staining of one additional tumour. This indicates that sialylation of T antigen is weak in this tumour type. These findings are in agreement with Seifert and Caselitz [15] using PNA. Studies with titration of the MAb HH8 directed to T antigen (data not shown) showed that the staining in carcinomas in pleomorphic adenomas disappeared at a higher dilution of the MAb than staining of the other types of carcinomas, indicating that more antigenic sites are present in carcinomas in pleomorphic adenomas, a finding which may have prognostic significance.

The tumours with participation of myoepithelial cells (i.e. glandular–tubular variant of adenoid cystic carcinomas and polymorphous low grade adenocarcinomas) differed from the other T and sialosyl-T antigen positive tumours, because myoepithelial cells in the tumours had retained a normal glycosylation pattern (i.e. expression of T and sialosyl-T antigens in the cytoplasm). The normal glycosylation pattern of these cells could be one of the reasons for the good prognosis for patients with these tumours [2].

The focal expression of mucin-type A antigen in all histological types of tumours from blood group A patients, except carcinomas in pleomorphic adenoma, is interesting, because loss of blood group A antigen is a well-known feature of carcinomas in the oral cavity and urinary bladder [25]. The presence of A antigen indicates that, although the tumours express an aberrant glycosylation pattern with accumulation of immature precursor structures, at least some of the cells have

retained a normal glycosylation pattern and that A enzyme necessary for the conversion of H to A antigen must be present [25].

Our results indicate that simple mucin-type T, sialosyl-T, H and A antigens could not contribute to classifying salivary gland carcinomas in new groups which are different from the classical histological types. However, all high and moderate differentiated carcinomas expressed mucin-type T, sialosyl-T, H and A antigens, but some heterogeneity was seen within the group of poorly differentiated tumours. Certain tumour types nearly always expressed T and sialosyl-T antigens, whereas other types were always negative. This is interesting in relation to the findings of Itskowitz [5] who showed that adenocarcinomas of the colon could be divided into different groups with prognostic significance, based on the expression of sialosyl-Tn (which is the sialylated precursor structure of T antigen). This new classification was independent of Duke's stage, tumour type and grade. A comparable relationship has been described in patients with bladder tumours. In these tumours, expression of T and sialosyl-T antigens and lack of these structures, respectively, can separate tumours with identical histological appearance into groups with high risk versus low risk of recurrence [26, 27].

Our findings that T antigens are accumulated in salivary gland carcinomas is also interesting because investigations have shown that T antigen is immunogenic in patients with cancer and that synthetic T antigen can be used as active immunotherapy and prolong the lives of mice with lethal breast cancer [3, 28]. Salivary gland carcinomas expressing T antigen may therefore also be a potential target for such a therapy.

In conclusion, we have elucidated the expression of simple mucin-type T (Thomsen–Friedenreich), sialosyl-T, H and A antigens in salivary gland carcinomas. The accumulation versus lack of expression of the investigated structures in poorly differentiated areas of the tumours is a finding which should be evaluated in prospective studies.


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