

**Glicosilação aberrante associada ao cancro de bexiga: avaliação do valor prognóstico**

**Abnormal glycosylation related to bladder cancer: Assessment of its prognostic value**

**Authors:** Paula A. Videira<sup>1</sup>, Inês F. Amado<sup>1,2</sup>, M. Correia<sup>1</sup>, Fernando M. Calais da Silva<sup>2</sup>, Fernando Calais da Silva<sup>2</sup>, Fabio Dall'Olio<sup>3</sup>, Dário Ligeiro<sup>4</sup>, Hélder Trindade<sup>1</sup>

**Affiliation of the authors:**

<sup>1</sup> Departamento de Imunologia FCM-UNL, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal.

<sup>2</sup> Grupo Português Génito Urinário

<sup>3</sup> Dipartimento di Patologia Sperimentale, Via S. Giacomo 14, 40126, Bologna, Italy

<sup>4</sup> Centro de Histocompatibilidade do Sul

**To whom correspondence should be addressed:**

Paula A. Videira, Departamento de Imunologia FCM-UNL, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal.

Telephone: +351 218 803 045; Fax: +351 218853480

Email: [pvideira.imuno@fcm.unl.pt](mailto:pvideira.imuno@fcm.unl.pt)

## RESUMO:

O carcinoma da bexiga (CB) apresenta uma elevada propensão para a recorrência ou progressão e esta tendência não está ainda totalmente elucidada. De facto, a terapêutica convencional falha em cerca de um terço dos doentes, principalmente devido a recorrência.

A identificação de marcadores mais credíveis para identificar oportunamente a progressão de CB e monitorizar melhor a administração da terapêutica complementar, é ainda premente.

Sabe-se que a superfície das células cancerosas é habitualmente aberrantemente glicosilada. Os antígenos T são estruturas glicosídicas, funcionalmente importantes para a progressão do cancro por influenciarem a adesão celular, incluindo o reconhecimento das células cancerosas por células imunes. Estes antígenos podem ser mascarados pelo ácido siálico (antígenos sialil-T), um processo desempenhado por sialiltransferases (STs), cujas consequências para a progressão do cancro não são claras e dependem do tipo de cancro. No caso do CB existem vários relatórios clínicos sobre a expressão dos antígenos T ou sialil-T, mas o papel da sialilação ou das STs ainda não foi esclarecido.

Analisámos tumores de doentes com CB e observámos que a expressão genética da ST, *ST3Gal.I*, se encontrava significativamente superexpressa em 75% dos casos. Os dados de expressão genética também indicaram a presença de células dendríticas, nas biópsias destes tumores, na sua maioria em estado imaturo. Esta presença está significativamente associada com a superexpressão da *ST3Gal.I*. Para complementar os nossos dados, estudámos a expressão do antígeno T em quatro linhas celulares de CB e encontramos uma correlação significativa entre a expressão de *ST3Gal.I* e de antígenos sialil-T superficiais. A transferência genética de cDNA de *ST3Gal.I* na linha celular HT1376, que expressava o antígeno T, permitiu que estas células expressassem antígenos sialil-T e confirmou o papel da *ST3Gal.I* na sialilação do antígeno T em CB.

Estes dados sugerem que o CB expressa antígenos sialil-T, essencialmente devido ao aumento da expressão do gene *ST3Gal.I* e estes antígenos podem desempenhar um papel na imunossupressão.

O nosso objectivo é aprofundar o estudo aqui apresentado, quer analisando amostras clínicas, quer comparando a malignidade das células de CB estabelecidas como expressando os antígenos T ou sialil-T. A exploração do papel destes antígenos na progressão do CB permitirá o desenvolvimento de terapêuticas mais convenientes e a avaliação de marcadores tumorais mais credíveis.

Abreviaturas: CB: Carcinoma da bexiga; ST: Sialiltransferases; CD Células dendríticas

## **Abstract**

Bladder cancer (BC) has an elevated propensity for recurrence or progression and this tendency is not completely elucidated. In effect, the standard treatments fail in almost one third of the patients, mainly due to recurrence. There is still a need for more reliable markers to identify opportunely the BC progression and to better monitor the administration of complementary therapy.

It is known that cancer cells surface is usually aberrantly glycosylated. The T antigens are glycosidic structures, which are functionally important in cancer progression by mediating the cell adhesion, including the recognition by immune cells. These antigens can be masked by sialic acid (sialyl-T antigens), a process carried out by specific sialyltransferases (STs), whose consequences for cancer progression are unclear and depend on the type of cancer. In BC, there are some clinical reports about the expression of the T or sialyl-T antigens, but the role of sialylation or STs remains to be elucidated.

We have analysed tumours from BC patients and observed that the gene expression of the ST, *ST3Gal.I*, was significantly overexpressed in 75% of the cases. The gene expression data also indicated the presence of dendritic cells (DCs), mostly in an immature state, within these tumour samples. This presence was significantly associated with the *ST3Gal.I* overexpression. In order to complement our data, we have studied the expression of the T antigen in four BC cell lines and we have found a significant correlation between *ST3Gal.I* and the surface expression of sialyl-T antigens. The genetic transfer of *ST3Gal.I* cDNA into the HT1376 cell line, which expressed the T antigen, enabled these cells to express sialyl-T antigens and confirmed the role of *ST3Gal.I* in the sialylation of the T antigen in BC. These data suggest that BC expresses the sialyl-T antigens, mainly due to the increased expression of *ST3Gal.I* gene and these antigens may have a role in immunosuppression.

Our aim is to continue further the work here presented, either analysing the clinical specimens, either comparing the malignancy of the established BC cells expressing the T or the sialyl-T antigens. The exploitation of the role of these antigens for BC progression will permit the development of more convenient BC treatment and assessment of more reliable tumour markers.

**Abbreviations:** BC: bladder cancer; ST: sialyltransferases; DC: dendritic cells;

## **Introduction**

Bladder cancer (BC) is one of the most common cancers in humans and its incidence has been increasing during the past years [1]. Approximately 70% of these cancers are presented as superficial (pTa, pT1, pTis) without causing death. Yet, superficial BC has a high recurrence rate after the initial treatment and it is becoming a public health problem and a social and economic burden. The standard treatments, such as resection followed by intravesical bacillus Calmette-Guérin (BCG), fail in almost one third of the patients, due to recurrence, tumour progression to a higher grade or intolerance to therapy [2]. In the case of intravesical BCG, the best schedule and dosage have not been well defined [3] and the management of BCG failures is also controversial. Moreover, it is necessary a frequent follow-up of the patients, performed with the current gold standard, cystoscopy with cytology, but both techniques are not perfect. Cystoscopy is invasive and does not detect completely multifocal lesions and cytology has low sensitivity for the detection of low grade lesions [4].

Given these negative aspects, efforts should be done to identify enhanced tumour markers to better monitor the administration of complementary therapy, to predict the patient response to therapy and to identify opportunely the progression of BC.

The achievement of these goals depends definitely on the exploitation of fully understood pathophysiological events critical for BC. But many of the mechanisms underlying BC are still undisclosed and therefore research should glance backward in order to fully understand the progression of this type of cancer.

Almost every type of cancer is characterized by the expression of deranged glycosidic structures on membrane or secreted glycoconjugates. These structures may regulate tumour proliferation, invasion, haematogenous metastasis and angiogenesis [5]. The glycosidic T antigen or Thomsen-Friedenreich antigen is a O-glycosidically linked structure (Gal $\beta$ 1,3GalNAc $\alpha$ 1) (Fig. 1), whose expression is largely increased in many types of cancers including kidney, prostate, cervix, ovary, breast, liver, stomach and colorectal carcinomas [6]. Its broad expression has guided some researchers to study their potential use as prognostic marker or in therapeutic approaches. According to recent data from the literature, these antigens are functionally important in cancer

progression by allowing increased interaction/communication of the tumour cells with endogenous proteins [7]. In fact, these antigens may bind circulating proteins, such as galectins, which in turn are involved in the regulation of the cell progression, adhesion and metastasis [8]. These antigens may also alter the cancer cell interaction with cells expressing galectins or other surface binding proteins. These interactions contribute to trigger defecting microvascular angiogenesis [9] and abrogate the recognition by the immune system cells [10]; thus, the expression of these antigens may represent a mechanism by which tumours escape immunosurveillance.

In some cancers, the T antigens are masked by the addition of a sugar, the sialic acid, giving rise to the sialyl-T antigens (Fig. 1). The masking (sialylation) is performed by sialyltransferases (STs), which are enzymes that transfer sialic acid and whose expression is often deregulated in cancer (reviewed in [11]). In the case of T antigens, three STs, ST3Gal.I, ST3Gal.II and ST3Gal.IV, are described as able to sialylated this antigen [11]. The consequences of sialylation in the ascribed functions of the T antigens in cancer progression are not fully understood. Apparently, the T or sialyl-T antigens are implicated in cancer progression in a cell or tissue type specific manner, and their contribution is much more complex, depending on their surface density and/or on the cell surface proteins.

The T antigens seems also to be particular important in BC and its expression has been correlated with a good prognosis by some studies [12, 13] but to a worse prognosis by others [14, 15]. In some specimens the T antigen is readily detectable, while in other specimens it is masked by sialic acid. Apart for the clinical reports, the contribution of the T or sialyl-T antigens for BC progression remains to be elucidated. Furthermore, there are no published data on the role played by STs in determining the type of T antigen expressed in BC cells.

In this study, we have investigated, in human BC specimens, the expression of STs putatively involved in the sialylation of T antigens. We have observed that the *ST3Gal.I* gene was significantly overexpressed in 75% of the cancers. Additionally, through the gene expression analysis of CD1c and HLA-DR, dendritic cell (DC) markers, we have found that HLA-DR was mostly repressed, but the expression of *ST3Gal.I* and *CD1c* genes was significantly associated, denoting the presence of DCs, mostly immature, within these cancer tissues. In addition, when studying BC cell lines, we observed a significant correlation between ST3Gal.I and the cell surface expression of sialyl-T antigens. The role of ST3Gal.I in the sialylation of T antigens was confirmed, through

the establishment of a BC cell line expressing a ST3Gal.I lentivirus construct. Our aim is to continue further the work here presented, either analysing the clinical samples, either comparing the malignancy of BC cells expressing the T or the sialyl-T antigens.

## **Methods**

### *Surgical specimens*

These studies included 32 patients with superficial BC. All patients were treated at the Urology Service, from Hospital do Desterro at Lisbon and submitted to transurethral resection of the cancer (TUR-B).

Samples from normal and tumour bladder tissue were obtained at surgery and stored in RNA later®. Informed consent was obtained from all patients.

### *Cell lines*

Four human BC cell lines, HT1374, 5637, MCR and T24 were routinely grown in Dulbecco's modified Eagle medium (DMEM), supplemented with foetal calf serum, glutamine, penicillin and streptomycin.

### *Generation of transductant bladder cancer cell lines with sialyltransferases constructs*

The whole coding region of ST3Gal.I gene (obtained from 5637 cell line cDNA) was inserted into a lentivirus vector (pLenti6/V5). The HT1376 cell line was then transduced with the obtained ST3Gal.I construct or the vector alone. Transduced cells were selected by antibiotic resistance to blasticidine.

### *Analysis of the phenotypic expression of TF antigen by flow cytometry*

Cells were first incubated in the presence or absence of neuraminidase (enzyme removing sialic acid) and then stained with *Arachis hypogaea lectin* (PNA) in order to analyse the surface expression of T antigens.

Cells were then analysed in a FacsCalibur Flow cytometer using CellQuest software (Becton Dickinson), following standard procedures.

### *Analysis of relative gene expression*

The expression of *ST3Gal.I*, *ST3Gal.II*, *ST3Gal.IV*, *HLA-DR* and *CD1c* genes was analysed by Real-Time PCR. Briefly, total RNA was extracted from tissues or cell lines

and reverse transcribed into cDNA. Real time PCR was performed by using the Taqman chemistry.

#### *Sialyltransferase assay*

Total ST activity was assayed in whole homogenates as previously described [16] with minor modifications, using Gal $\beta$ 1,3GalNAc $\alpha$ 1-O-benzyl (benzyl-T antigen) as acceptor.

#### *Statistical analysis*

Data was analysed for statistical significance using the Student's t-test and ANOVA One-way (GraphPad Prism Version 4.0). Correlations between variables were tested using the Spearman and Pearson methods.

## **Results**

### *Sialyltransferases expression is altered in bladder cancer tissue*

We have studied the gene expression of the sialyltransferases (ST), mainly involved in the sialylation of the TF antigens (*ST3Gal.I*, *ST3Gal.II* and *ST3Gal.IV*), in samples of bladder tumours relative to normal urothelium. Among the ST genes analysed, the expression of the *ST3Gal.I* gene was statistically altered in BC ( $p < 0,001$ , based on the mean and median values) and overexpressed in 75% of the cancers (Fig. 2).

These results confirm that similarly to other type of cancers [11], the BC tissue also presents a deregulated expression of STs. In addition, the increased expression of *ST3Gal.I* gene suggests that the sialylated form of T antigens, the sialyl-T, was being expressed in the patient's tumours.

### *Bladder cancer cell lines express T and sialyl-T antigens*

In order to investigate further whether the T antigens were expressed in BC, we have analysed four human BC cell lines (HT1374, 5637, MCR and T24). Due to experimental constrains, we have opted to analyse the phenotypic expression of T antigens in bladder cell lines, rather than in patient tissues. Additionally, this analysis is aimed to identify cell lines expressing the non sialylated form of T antigen to be used in further genetic manipulation experiments.

The analysis included the cell staining using PNA lectin (recognizing the T antigen), the total ST activity towards benzyl-T antigen and the relative *ST* gene expression in these cell lines.

According to the PNA cell staining, it was evident the T antigen is expressed at high levels by two cell lines, HT1376 and MCR, but only weakly by 5637 cells and

intermediate by T24. After neuraminidase (enzyme removing sialic acid) treatment part of the 5637 and T24 become PNA positive, indicating that these cell lines express the T antigen masked by sialic acid (Fig. 3). In accordance with these results, HT1376 cells present low level of ST activity towards T antigen and 5637 cells showed the highest activity (Fig. 4). Both MCR and T24 cell line presented an intermediate ST activity. To investigate which gene could be responsible for the observed ST activity, we have analysed the expression of the *ST3Gal.I*, *ST3Gal.II* and *ST3Gal.IV* genes, described as involved in the T antigen sialylation, in the four cell lines (Fig. 5). Among the three genes, the expression of *ST3Gal.I* in the analysed cells gave the best correlation with the observed ST activity ( $r = 0,99$ ;  $p = 0,001$ ) which in turn was in accordance with the PNA staining. The data propose the *ST3Gal.I* gene as the major responsible for the sialylation of T antigens in BC. These findings together with the results obtained from patient's BC tissue, reinforce the idea the sialyl-T antigens were overexpressed whenever an increased expression of the *ST3Gal.I* gene was observed.

#### *Overexpression of ST3Gal.I gene in HT1376 bladder cancer cell line*

To better demonstrate the *ST3Gal.I* gene is involved in the sialylation of the T antigen in BC cells, we intended to manipulate its expression in a cell expressing non sialylated T antigens. To say, we expected the introduction of the *ST3Gal.I* coding region, in a cell presenting low levels of expression of this gene and expressing non sialylated T antigens, could alter the expression into the sialylated form of T antigen. According to the previous analysis, the HT1376 cell line fulfils this requisites and it was used in transduction experiments with a *ST3Gal.I* lentivirus construction or with the vector alone. The HT1376 transduced cells were first selected based on the acquired antibiotic resistance to blasticidine, an antibiotic resistance conferred by the lentivirus vector, and checked for the ST activity towards the benzyl-T antigen. When compared with the cells transduced with the vector alone, the *ST3Gal.I* transduced cells presented an almost twenty fold increased ST activity (from 8,69 to 171,33 nmol/h\* $\mu$ g protein), towards the benzyl-T antigen. According to the PNA cell staining (recognizing the T antigen), half of the *ST3Gal.I* transduced cells exhibited lower levels of T antigens (Fig. 6). Knowing that the untransduced HT1376 cells present high level of T antigens (Fig. 3), these results are indicative that in these cells, the T antigens are masked by sialic acid.

In addition, the data make evident the *ST3Gal.I* gene is involved in the expression of sialylated forms of T-antigen in BC.

### *Immature dendritic cell markers are identified in bladder cancer tissue*

It is known that DCs may accumulate within some cancers [17, 18]. Inside the tumours, DCs are usually unable to mature and as a result they are incapable to induce a correct immune response and instead they induce tumour tolerance [19]. Nevertheless, in some tumours, mature DCs may be observed at the peritumoural area [17]. In order to investigate whether DCs accumulate also in BC tissue, we have analysed the referred specimens of BC tissue for the expression of CD1c and HLA-DR genes. We were able to detect the expression of CD1c gene, coding for a specific DC marker, in 84% of the analysed specimens (Fig. 7). Among them, only 40% (34% of the total samples) also expressed HLA-DR gene, an antigen presentation molecule, expressed by antigen presenting cells, such as mature DCs.

Although this assay cannot discriminate infiltrated DCs from peritumoural DCs, it does give an indication of the presence of immature DCs in BC tissue.

In two recent studies, it was reported that tumour-associated glycosidic antigens were able to abrogate the maturation of infiltrating DCs [20, 21]. So as to determine a putative association between the expression of T or sialyl-T antigens and the presence of mature or immature DC, we searched for a correlation between the increased expression of *ST3Gal.I* gene (in tumour relative to urothelium) and the expression of immature DCs markers (presence of CD1c and absence of HLA-DR expressions). In this study we have observed no significant correlation between both parameters. Nevertheless, we have found a significant correlation between the levels of mRNA of *ST3Gal.I* and *CD1c* ( $p < 0,001$ , Person correlation) (Fig. 7).

### **Discussion**

The aberrant expression of T antigens observed in various types of cancer interferes with the interaction between the tumour cell and other cells or soluble proteins, altering, among other aspects, its potential to grow, to form metastasis and their immunogenicity, [7]. How the expression of these antigens affects the cancer progression depends considerably on the type of cancer tissue and whether the antigens are capped by sialic acid (sialyl-T antigens). This sialylation depends on the action of specific sialyltransferases (STs) that transfer the sialic acid into the T antigen.

This study is the first analysing, in BC specimens, the expression of STs known to be able to sialylate the T antigen. In most cases the expression of these STs is altered, compared with normal urothelium and the ST3Gal.I presented a significant increase, in 75% of the BC tested. Further assays are envisaged in order to elucidate whether this increase is associated with the presence of sialyl-T antigens in the cancer tissue. Nevertheless, the use of BC cell lines allowed us to confirm the involvement of ST3Gal.I in the synthesis of sialyl-T antigens in BC cells. In fact, the HT1376 cell line, when transduced with the ST3Gal.I construct, expressed lower levels of the T antigen, as shown by the PNA lectin binding assay, suggesting these antigens were masked by sialic acid or, in other words the transduced cells were expressing sialyl-T antigens. However, as this expression was only manifested in 50 % of the cells, we intend to sort the ST3Gal.I transduced cells in order to get individual clones with higher content of sialyl-T antigens.

The establishment of HT1376 cell lines variants, expressing the T or the sialyl-T antigens, is an excellent model to further compare the involvement of these antigens in BC progression.

Additionally, we evaluated the gene expression of dendritic cell (DC) markers in BC specimens and we observed that the great majority expressed *CD1c*. These results suggested DCs accumulate within this cancer, which is in accordance with the observations made in other types of cancers [17, 18]. However, most of the analysed BC showed no expression of *HLA-DR*, the class II major histocompatibility complex, signifying those DCs haven't undergone maturation. Within colorectal cancer, DCs are usually unable to mature and their capability of inducing an immune response is downregulated [19]. In recent studies, it was reported that tumour-associated glycosidic antigens were able to abrogate the DC function and lead to tolerance induction to colorectal cancer [21]. According to the gene expression analysis data, we found a correlation between the mRNAs level of *ST3Gal.I* and *CD1c*, suggesting a parallel between the presence of sialyl-T antigens and DCs. Nevertheless, further studies, involving techniques such as the microscopic evaluation, to co-localize tumour cells expressing these antigens and DCs in BC tissue are mandatory to confirm these assumptions.

In summary, this study evidences the sialyl- T and T antigens are present in BC due to the aberrant expression of ST3Gal.I. So far, it was not possible to relate the data obtained from BC patients with their clinical outcome. The patient's data indicate,

however, an association between the expression of these antigens and DC based immunosuppression. The establishment of the cell line variants will allow us to continue these studies and evaluate whether the presence of the T or the sialyl-T antigens at cell surface modulate the malignant phenotype of BC.

If these antigens are proven to modulate the pathophysiological events critical for BC progression, new therapeutical approaches could be designed based on these findings. For instance, molecules or antibodies could be studied in order to avoid the interactions between the tumour cell and other cells or molecules, such as galectins. This will be quite valuable to delay angiogenesis, adhesion, immune suppression, etc. The development of new therapeutics targeting glycosidic antigens is now emerging. As an example, in breast cancer, the tumour vaccine *Theratope*, is based on the sialyl-Tn antigen (another member the Thomsen-Friedenreich antigen family, highly expressed in breast cancer) and it was proven to have a positive survival effect in breast cancer patients [22]. We have also verified that the (sialyl-) T antigens are easily detected in urine by simple techniques such as lectin dot blot analysis. Therefore the potential use of these antigens as prognostic markers to better identify the progression of bladder cancer, or to monitor the administration of therapy should be explored.

### **Acknowledgements**

This work was partially supported by Astellas Pharma (Yamanuchi Price). We acknowledge University of Bologna for supporting stay of P. A. V. at this University (Early Stage Research Fellowship from Institute Advanced Study) and Leonardo da Vinci European Program for I. F. A. fellowship. We thank João Sobral for his help in organizing the patient's data.

## **References**

1. Lamm DL, McGee WR, Hale K. Bladder cancer: Current optimal intravesical treatment. *Urol Nurs* 2005; 25:323,6, 331-2.
2. Tishler M, Shoenfeld Y. BCG immunotherapy--from pathophysiology to clinical practice. *Expert Opin Drug Saf* 2006; 5:225-9.
3. Witjes JA. Management of BCG failures in superficial bladder cancer: A review. *Eur Urol* 2006; 49:790-7.
4. Witjes JA, Moonen PM, van der Heijden AG. Comparison of hexaminolevulinate based flexible and rigid fluorescence cystoscopy with rigid white light cystoscopy in bladder cancer: Results of a prospective phase II study. *Eur Urol* 2005; 47:319-22.
5. Fuster MM, Esko JD. The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nat Rev Cancer* 2005; 5:526-42.
6. Brockhausen I. Pathways of O-glycan biosynthesis in cancer cells. *Biochim Biophys Acta* 1999; 1473:67-95.
7. Yu LG. The oncofetal thomsen-friedenreich carbohydrate antigen in cancer progression. *Glycoconj J* 2007; .
8. Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. *Nat Rev Cancer* 2005; 5:29-41.
9. Xia L, Ju T, Westmuckett A, et al. Defective angiogenesis and fatal embryonic hemorrhage in mice lacking core 1-derived O-glycans. *J Cell Biol* 2004; 164:451-9.
10. Sotiriadis J, Shin SC, Yim D, Sieber D, Kim YB. Thomsen-friedenreich (T) antigen expression increases sensitivity of natural killer cell lysis of cancer cells. *Int J Cancer* 2004; 111:388-97.
11. Dall'Olio F, Chiricolo M. Sialyltransferases in cancer. *Glycoconj J* 2001; 18:841-50.
12. Dobrowolski ZF, Dus D, Halasa J, Radzikowski C. Prognostic value of an assessment of ABH(0) isoantigens and thomsen-friedenreich (TF) antigen expression in patients with urinary bladder tumours. *Int Urol Nephrol* 1995; 27:395-404.
13. Dow JA, di Sant'Agnese PA, Cockett AT. Expression of blood group precursor T antigen as a prognostic marker for human bladder cancer treated by bacillus calmette-guerin and interleukin-2. *J Urol* 1989; 142:978,81; discussion 981-2.
14. Langkilde NC, Wolf H, Clausen H, Orntoft TF. Human urinary bladder carcinoma glycoconjugates expressing T-(gal beta(1-3)GalNAc alpha 1-O-R) and T-like antigens: A comparative study using peanut agglutinin and poly- and monoclonal antibodies. *Cancer Res* 1992; 52:5030-6.
15. Langkilde NC. T-antigens in primary non-invasive and superficially invasive human urinary bladder tumors: The correlation to tumor recurrence and tumor progression. A mini-review. *Scand J Urol Nephrol Suppl* 1995; 172:45-9.

16. Dall'Olio F, Malagolini N, Guerrini S, Lau JT, Serafini-Cessi F. Differentiation - dependent expression of human beta-galactoside alpha 2,6-sialyltransferase mRNA in colon carcinoma CaCo-2 cells. *Glycoconj J* 1996; 13:115-21.
17. Bell D, Chomarat P, Broyles D, et al. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J Exp Med* 1999; 190:1417-26.
18. Suzuki A, Masuda A, Nagata H, Kameoka S, Kikawada Y, Yamakawa M, Kasajima T. Mature dendritic cells make clusters with T cells in the invasive margin of colorectal carcinoma. *J Pathol* 2002; 196:37-43.
19. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 2000; 192:1213-22.
20. Rughetti A, Pellicciotta I, Biffoni M, et al. Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells. *J Immunol* 2005; 174:7764-72.
21. Gijzen K, Broers KM, Beeren IM, Figdor CG, Torensma R. Binding of the adhesion and pathogen receptor DC-SIGN by monocytes is regulated by the density of lewis X molecules. *Mol Immunol* 2007; 44:2481-6.
22. Ibrahim NK, Murray JL. Clinical development of the STn-KLH vaccine (theratope). *Clin Breast Cancer* 2003; 3 Suppl 4:S139-43.

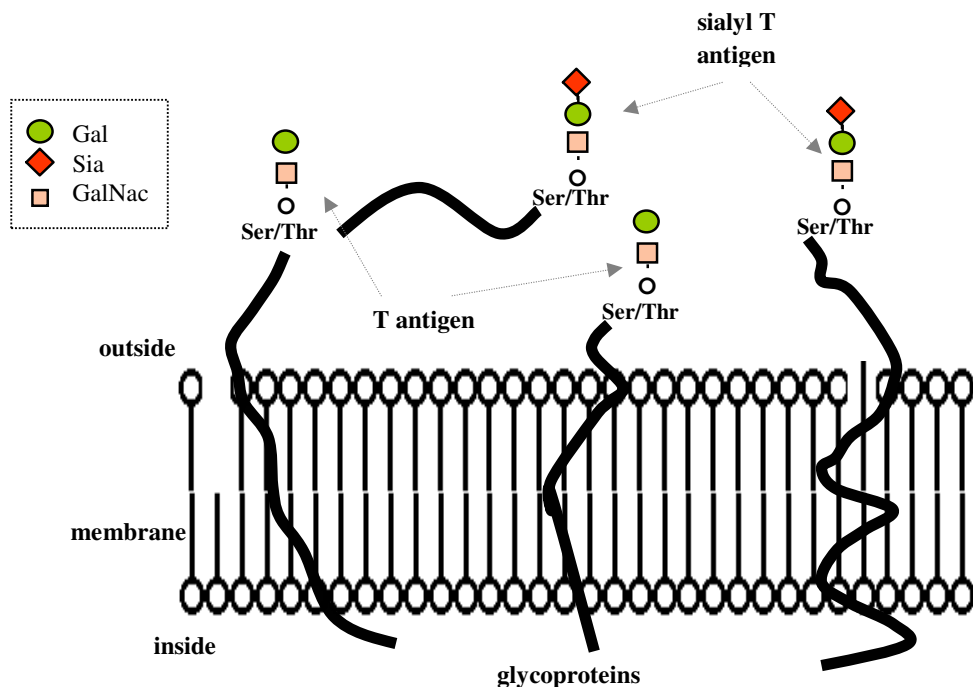


Fig. 1. Simplified representation of the biosynthesis of T (Thomsen-Friedenreich) antigens. The T antigen is comprised of a O-linked galactosyl N-acetylgalactosamine ( $\text{Gal}\beta 1,3\text{GalNAc}\alpha 1$ ) and it can be elongated by the addition of a sialic acid  $\alpha 2,3$ -linked to galactose, forming the sialyl-T antigen.

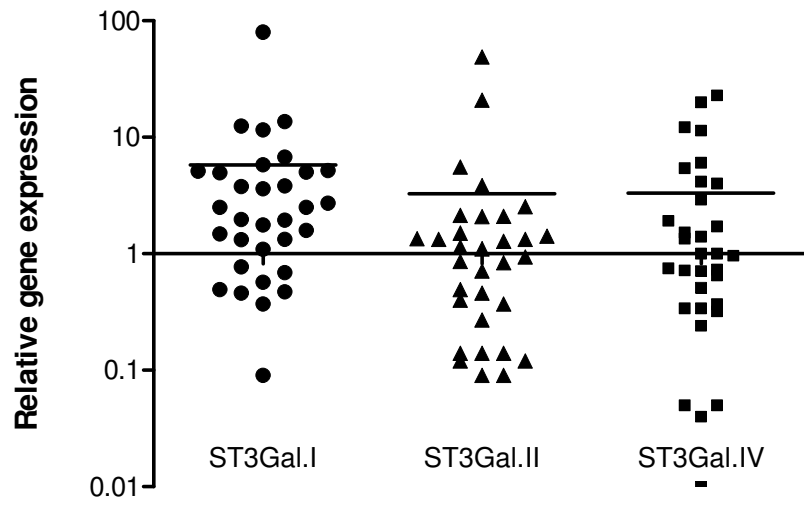


Fig. 2. Relative expression of the sialyltransferase genes involved in the sialylation of the T antigen, *ST3Gal.I*, *ST3Gal.II* and *ST3Gal.IV*, tested in 32 samples of bladder tumour tissue relative to normal urothelium. Values above or below 1 indicate, respectively, overexpression or downregulation of the specific gene.

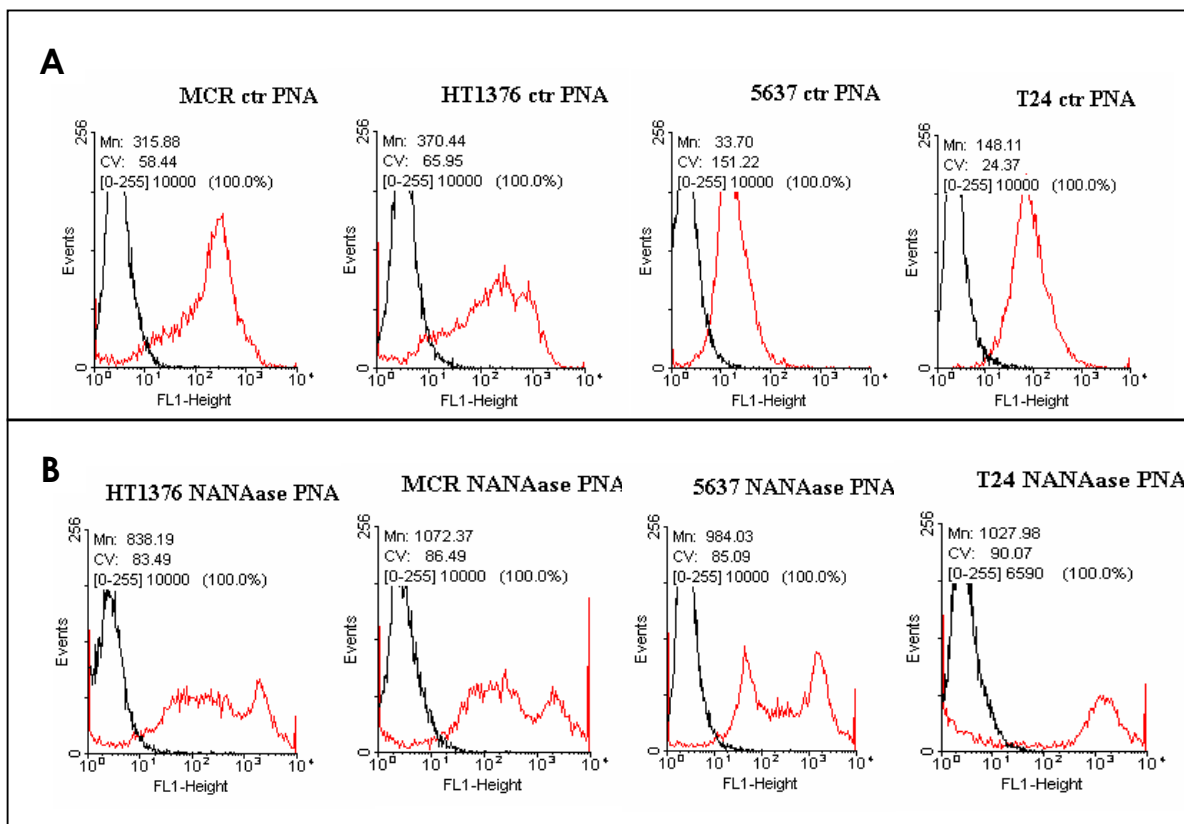


Fig. 3. Flow cytometry analysis with PNA lectin (recognizing the T antigens) of bladder cancer cell lines before (A) an after (B) neuraminidase treatment (NANAase-enzyme removing sialic acid).

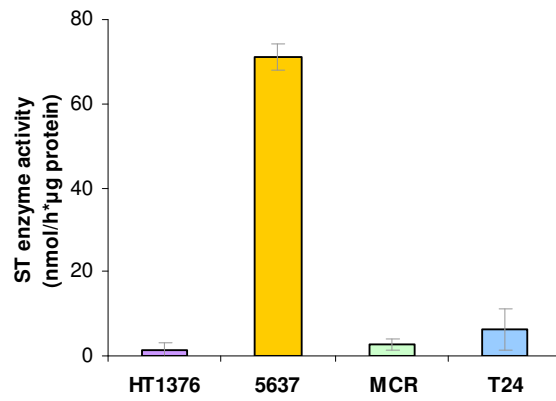


Fig. 4. Total sialyltransferase activity towards benzyl-T antigen in HT1376, 5637, MCR and T24 bladder cancer cell lines.

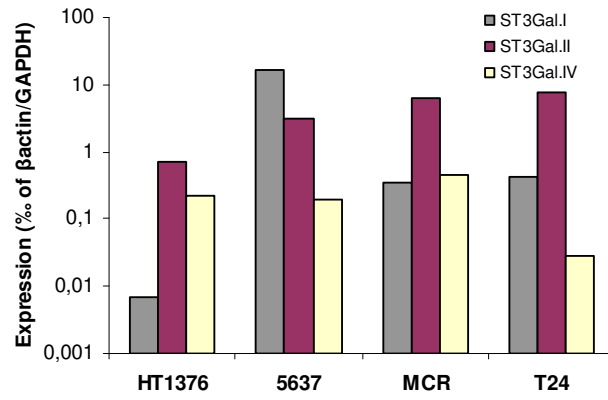
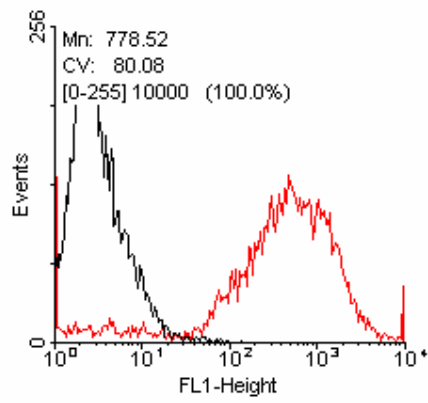


Fig. 5. Relative mRNA level of *ST3Gal.I*, *ST3Gal.II* and *ST3Gal.IV* in HT1376, 5637, MCR and T24 bladder cancer cell lines.

A HT1376 ctr PNA



B HT1376/ ST3Gal.I PNA

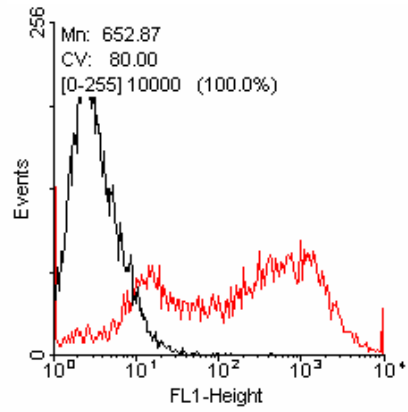


Fig. 6. Flow cytometry analyses with PNA lectin (recognizing the T antigens) of the HT1376 cells transduced with the vector alone (A) or the ST3Gal.I construct (B).

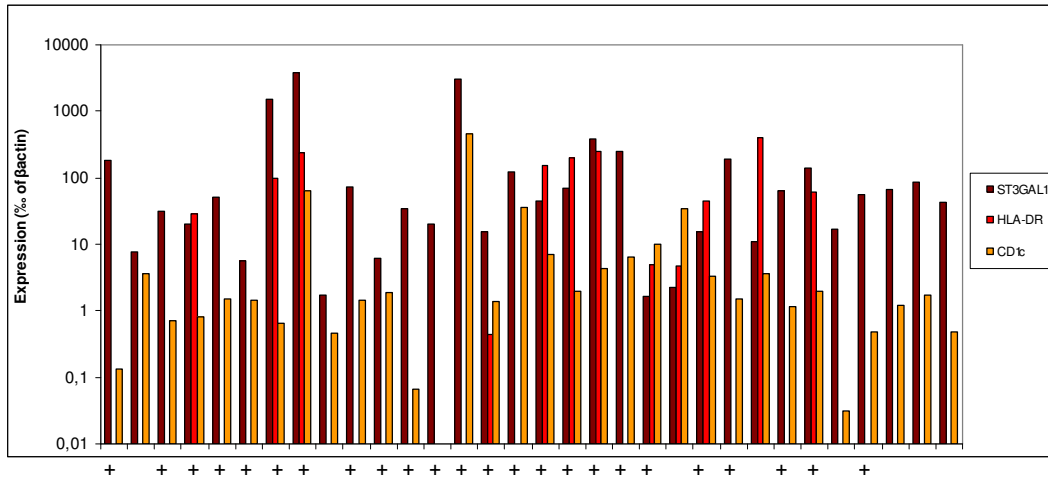


Fig. 7. Relative mRNA level of *ST3Gal.I*, *HLA-DR* and *CD1c* in 32 samples of bladder cancer patients. Samples, where the expression of *ST3Gal.I* gene was overexpressed relative to normal urothelium, are indicated by +.