Universidade de Aveiro | Departamento de Química

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Gonçalves Santos

Relevância clínica do antigénio sialil-Tn no cancro da bexiga

Clinical relevance of the sialyl-Tn antigen in bladder cancer

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Bioquímica Clínica, realizada sob a orientação científica do Professor José Alexandre Ferreira, Investigador de Pós-Doutoramento do Centro de Investigação do Instituto de Oncologia do Porto (IPO-Porto) e do Departamento de Química da Universidade de Aveiro

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À minha mãe

(*in memoriam*)

o júri

Presidente

Professora Doutora Rita Maria Pinho Ferreira

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palavras-chave cancro da bexiga, sialil-Tn, invasão, metastização, xenógrafos, biomarcador de prognóstico

resumo

Aproximadamente 50% dos doentes com carcinomas musculo invasivos de bexiga (MIBC) desenvolvem metástases num período de 5 anos após a cirurgia, mesmo quando submetidos a regimes de quimioterapia pré- e pós-cirurgia. Assim, surge a necessidade de se desenvolverem biomarcadores específicos para identificar fenótipos celulares agressivos e terapias diretas baseadas em marcadores moleculares. Recentemente, surgiram evidências de que tumores de bexiga em estadio avançado expressam sialil-Tn (STn) na superfície celular, um antigénio associado a tumores que pode ser usado para detetar fenótipos agressivos no cancro da bexiga. Este antigénio resulta de uma paragem prematura na *O*-glicosilação de proteínas presentes na superfície celular, e tem demonstrado ser capaz de prevenir o reconhecimento imunológico das células cancerígenas, evitando a eliminação das células metastáticas, modular o fenótipo maligno e induzir a capacidade metastática destas mesmas células. No presente estudo, avaliou-se por imunohistoquímica a expressão de STn e Ki-67 (marcador de proliferação celular) numa série de 96 doentes com cancro de bexiga em diferentes estadios. Este estudo demonstrou uma associação entre a expressão de STn e a proliferação tumoral e invasão. A expressão de STn também foi observada em metástases ganglionares e à distância de tumores STn positivos. O antigénio STn foi maioritariamente observado em glicoproteínas de alto peso molecular (>250 kDa) e em proteínas de baixo peso molecular a 25, 15 e 10 kDa. Estas espécies de baixo peso molecular predominaram nas metástases ganglionares que também não apresentaram proteínas de alto peso molecular, sugerindo uma assinatura molecular associada com metastização. De modo geral, o antigénio STn apresenta potencial para o desenvolvimento de novas terapias contra tumores de bexiga agressivos. Estudos futuros deverão ser realizados para determinar a natureza das glicoproteínas que expressam o STn e confirmar a possibilidade de uma assinatura molecular associada com metastização.

A segunda parte do trabalho focou-se na validação de um xenógrafo de tumor de bexiga expressando STn, como modelo para teste de fármacos e identificação de biomarcadores de prognóstico. Um tumor de bexiga musculo invasivo e STn positivo e os xenotransplantes resultantes da primeira, segunda e terceira passagens foram comparados em relação a proliferação (Ki-67), diferenciação (p63 e CK20) e expressão de STn, por imunohistoquímica. Os padrões de histologia e imunohistoquímica entre o tumor primário e os xenógrafos eram idênticos, revelando um grau de similaridade entre o modelo animal e o tumor humano. Contudo, os níveis de p53 e Ki-67 aumentaram ao longo das passagens enquanto os níveis de STn diminuíram, sugerindo uma seleção dos clones mais proliferativos.

Estas observações possuem uma importância fundamental na expansão do conhecimento sobre a relevância clínica do STn no cancro da bexiga e na criação dos fundamentos para uma terapia baseada neste antigénio.

keywords bladder cancer, sialyl-Tn, invasion, metastasis, xenografts, prognostic biomarker

abstract

Approximately 50% of muscle invasive bladder cancers (MIBC) develop metastasis within 5 years after surgery, despite being subjected to pre- and post-surgery chemotherapy regimes. Thus, specific biomarkers to target aggressive cell phenotypes and direct molecular-based therapy are warranted. Recently, evidences have been presented that advanced stage bladder cancers express the cell-surface tumor-associated carbohydrate antigen sialyl-Tn (STn), which may be used to target aggressive bladder cancer cells. The STn antigen results from a premature stop in the *O*-glycosylation of cell-surface proteins and has been found to prevent immune recognition, contributing to avoid metastatic cell elimination, modulates the malignant phenotype and enhances the metastatic ability of cancer cells. In the present study, a series of 96 patients with bladder cancer of different stages was screened for STn expression and proliferation (over-expression of Ki-67) by immunohistochemistry. This showed an association between STn expression and tumor proliferation and invasion. STn expression was also observed in lymph node and distant metastases of STn positive tumors. The STn antigen was mainly detected in high-molecular weight glycoproteins (>250 kDa) and low-molecular weight proteins at 25, 15 and 10 kDa. These low-molecular weight species predominated in lymph node mestastasis samples that also did not present high-molecular weight proteins, suggesting a molecular signature associated with metastasis. Altogether, the STn antigen presents potential for the development of new therapies against aggressive bladder cancer. Studies should be conducted to determine the nature of the STn-expressing glycoproteins and disclosing the possibility of a molecular signature associated with metastasis.

The second part of the work focused on the validation of a STn-expressing bladder cancer xenograft as a model to drug testing and identification of prognostic biomarkers. A STn-positive muscle-invasive bladder tumor and its first, second and third generation xenotransplants were compared in relation to proliferation (Ki-67), differentiation (p63 and CK20), aggressiveness (p53) and STn expression, by immunohistochemistry. Histological and histochemical expression patterns were similar between primary tumor and xenografts, highlighting a degree of similarity between the animal model and the human tumor. However, p53 and Ki-67 levels increased along passages while STn decreased, suggesting a selection of the most proliferative clones.

The generated information is regarded of primary importance to expand the knowledge about the clinical relevance of STn in bladder cancer and create the rationale for a STn-based therapy.

A ignorância afirma ou nega veementemente. A ciência duvida.

Voltaire

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Abbreviations

β3/4 Gal-T	β3/4-galactosyltransferase
β3/4 Gn-T	*N*-β3/4-acetylglucosaminyltransferase
BCG	*Bacillus Calmette-Guérin*
C1Gal-T	Core 1 β(1-3) galactosyltransferase
C2Gn-T	Core 2 β(1-6)-*N*-acetylglucosaminyltransferase
C3Gn-T	Core 3 β(1-3) *N*-acetylglucosaminetransferase
CIS	Carcinoma *in situ*
Cosmc	Core 1 β(1-3) galactosyltranferase-specific molecular chaperone
ER	Endoplasmatic reticulum
Fuc-T	Fucosyltransferase
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
IHC	Immunohistochemistry
MIBC	Muscle invasive bladder cancer
MoAb	Monoclonal antibody
Neu5Ac	*N*-acetyl neuraminic acid
NMIBC	Non-muscle invasive bladder cancer
ppGalNAc-T	UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase
Ser	Serine
ST3Gal	α2,3-sialyltransferase
ST6GalNAc	GalNAc α2,6-sialyltransferase
STn	Sialyl-Tn antigen
Thr	Threonine
UDP-GalNAc	Uridine diphosphate – *N*-acetylgalactosamin

Chapter I | Introduction

Bladder cancer – clinicopathological classification, therapeutic challenges and the sialyl-Tn antigen

Urinary bladder cancer is the fifth most frequent neoplasia in western countries (and ninth worldwide), and has the highest treatment costs per patient of all cancers [1–3]. High recurrence rate and long-term follow-up, as well as repeated interventions, are the major causes that turn bladder cancer the costliest to treat among all solid tumors [2, 4].

Most of diagnosed bladder cancer cases in western countries (70-80%) are superficial non-muscle invasive carcinomas (NMIBC) that do not reach the *muscularis propria* [3–5]. NMIBC cases include papillary low-grade tumors confined to the mucosa (pTa), high-grade papillary lesions, that may invade the subepithelial connective tissue but not the muscle (pT1) or high-grade flat lesions termed carcinoma *in situ* (CIS), as shown in **Figure 1** [6–8]. High-grade tumors present poorly differentiated cells and higher aggressiveness, while low-grade tumors exhibit well differentiated cells. The standard treatment for NMIBC is complete transurethral resection (TUR) [4, 5, 7]. Approximately 10-30% of the patients with NMIBC are at an high risk of recurrence that may be accompanied by muscle invasive disease (MIBC), correlated with poor prognosis [3, 9]. The risk of recurrence and/or progression is determined by clinicopathological features – according to the European Organization for Research and Treatment of Cancer (EORTC), this group includes high-grade papillary tumors, CIS and multifocal disease [5, 7]. To decrease the risk of recurrence/progression, these patients are submitted to schedule of intravesical instillations with attenuated strains of *Mycobacterium bovis* (the *Bacillus Calmette-Guérin* (BCG) vaccine) [3, 4], after removal of the tumor. This treatment is known to promote a strong immunologic response in the bladder that ultimately contributes to eliminate the residual tumor [7, 10]. Despite effective in delaying recurrence, one third of the patients either do not respond or present intolerance to BCG, 70% of the responders relapse within 5 years and approximately 15% progress to muscle invasive disease [7, 8]. Moreover, many patients develop chronic cystitis or other side-effects during and after BCG therapy [3]. Upon therapeutic failure and/or muscle invasion, the patient is generally appointed for cystectomy [4, 5]. Therefore, alternative organ-spearing therapeutic options for NMIBC patients that either do not respond or show significant intolerance to BCG immunotherapy are warranted.

The remaining 20-30% of the newly diagnosed cases worldwide are muscle invasive bladder cancers (MIBC), that comprise pT2-pT4 stages (**Figure 1**) [3, 4]. These neoplasias are treated by radical cystectomy [3, 5], and also submitted to pre-and post-cystectomy neo-adjuvant chemotherapy, to reduce the risk of metastasis. However, the current chemotherapy regime carries significant toxicity, and approximately 50% of MIBC cases develop metastasis within 5 years [5, 11].

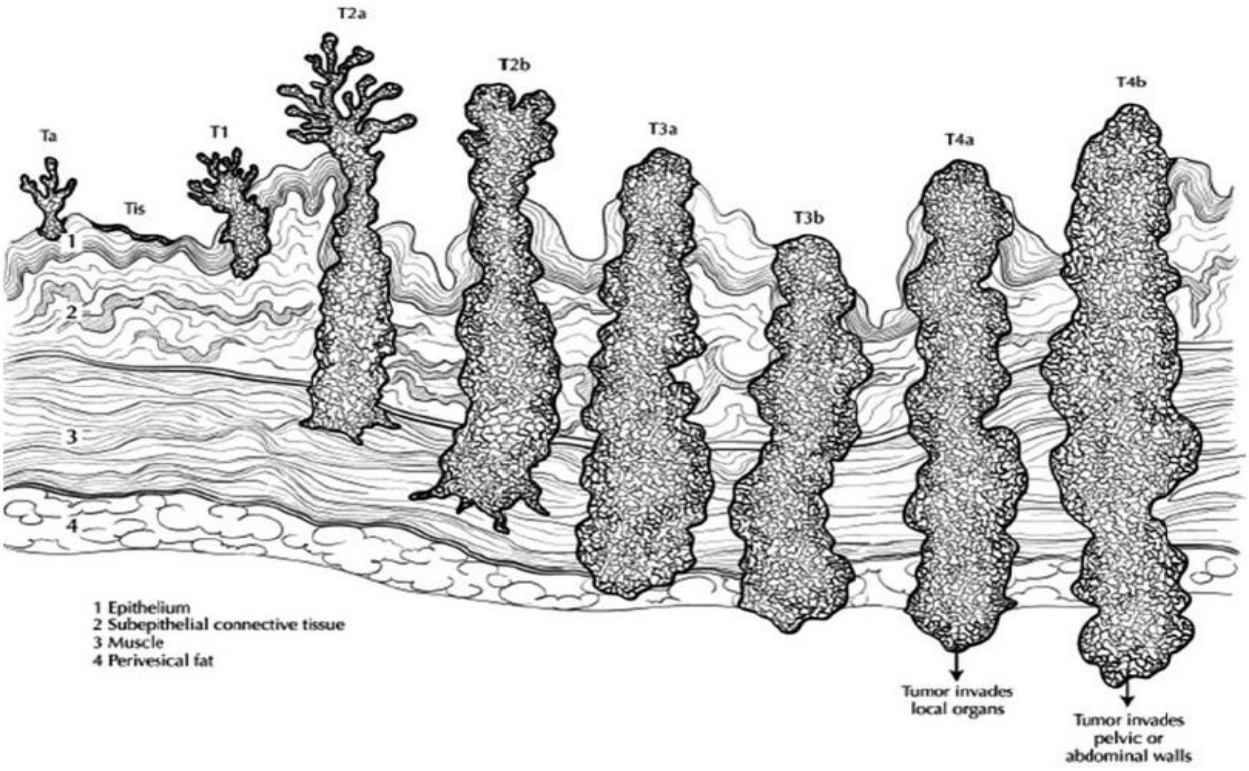


Figure 1 | Extension of the different stages of bladder cancer (NMIBC and MIBC) (reprinted from [4]).

At the moment, there is a lack of specific biomarkers to target aggressive cell phenotypes in bladder tumors, predict MIBC response to chemotherapy or assist the design of optimal treatment schemes, which would translate in better outcomes and improved overall survival. Therefore, the identification of these biomarkers is considered of main importance, since they may be used to avoid preventive cystectomy and/or reduce the chance of poor outcome, greatly improving the management of bladder cancer [3, 11].

Recent studies have pointed out that advanced stage NMIBC and MIBC express the sialyl-Tn (STn) antigen, a cancer-associated glycan that results from a premature stop in

the *O*-glycosylation of cell-surface proteins [11–13]. This antigen is not present in the healthy urothelium, denoting a cancer-specific nature, and has been found to promote the invasive and migration potential of bladder cancer cells *in vitro* [11]. Given its cell-surface nature, STn offers potential to target aggressive bladder cancer cells. However, despite these preliminary evidences, the clinical relevance of these alterations, such as the association with invasion and metastasis, remains to be verified. Additionally, the proteins carrying this alteration remain unknown. Thus, the confirmation of the association of the sialyl-Tn antigen with invasion and metastasis is warranted in order to establish the rationale for a novel therapeutics. The development of animal models expressing this antigen would also greatly benefit such a goal.

Protein *O*-glycosylation in eukaryotic cells

The sialyl-Tn (STn) glycan can be found as a post-translational modification of *O*-glycoproteins at the cell-surface of cancer cells. Glycosylation is the most common post-translational modification of proteins and plays a pivotal role in structural and functional features of these molecules – in fact, virtually, all proteins can be glycosylated [14, 15]. Glycans are more complex and diverse in structure and in composition than proteins. Furthermore, the glycosylation of proteins is not a template driven event and frequently the same protein may assume different glycoforms, making of glycan characterization a challenging analytical task [16, 17]. Moreover, glycan structures may vary significantly in response to changes in physiological conditions, with implications in cell-cell adhesion, cell recognition, activation/modulation of immune response and the activation of several intracellular signaling pathways [17, 18].

Protein *O*-glycosylation is a stepwise pathway that begins in the Golgi apparatus of eukaryotic cells, in which monosaccharides are added individually and sequentially to serine (Ser) and threonine (Thr) residues of proteins by a complex set of enzymes [15, 19]. *O*-glycans are highly expressed in mucins, a class of heavily glycosylated proteins secreted by mucosa and some exocrine glands [14, 20]. The high content in VNTR (*Variable Number Tandem Repeat*) regions exhibited by mucins, which are rich in Ser and Thr residues, provides the necessary backbone for a bulk of *O*-glycosylation [14, 20, 21]. As a result of its association with mucins, cell surface *O*-glycans are generally designated as mucin-type *O*-glycans; nevertheless, *O*-glycans can also be found in many other cell surface glycoproteins exhibiting Ser and Thr residues [14]. A certain variation of *O*-glycosylation seems to also occur in the nucleus and cytosol, a process termed nuclear *O*-glycosylation. This nuclear *O*-glycosylation is performed by a single *N*-acetylglucosamine (GlcNAc) residue that binds to a serine (Ser)/threonine (Thr), and appears to perform a signaling role similar to protein phosphorylation [22, 23]. In addition to mucins, a recent proteomic study has described almost 3000 glycosites in over 600 *O*-glycoproteins, 80% of each from the cell surface, but also from the cytoplasm and nucleous. This work greatly expanded the view of the *O*-glycoproteome and the array of protein function it may regulate [24].

Mucin-type *O*-glycans synthesis begins with the transfer of an α-*N*-acetylgalactosamine (GalNAc) from the donor-nucleotide sugar uridine diphosphate – *N*-acetylgalactosamine (UDP-GalNAc) to the hydroxyl group of a residue of Ser//Thr within the glycoprotein being synthesized [14, 15, 19]. This reaction forms the simplest mucin *O*-glycan – the Tn antigen (GalNAcα-*O*-Ser/Thr). This structure can be sialylated at *O*-6

position by a ST6GalNAc-I sialyltransferase, originating the sialyl-Tn (STn, Neu5Acα2-6GalNAcα-*O*-Ser/Thr) antigen; nevertheless, sialyl-Tn cannot function as substrate for any other glycosyltransferase, and thus the elongation of oligosaccharide chain stops (**Figure 2**) [14, 18, 20].

The initial step of *O*-glycosylation is catalyzed by a family of 20 membrane-bound enzymes denominated UDP-GalNAc:polypeptide glycosyltransferases (ppGalNAc-Ts), that have distinct but overlapping specificities [15, 19]. This family of enzymes has a C-terminal lectin domain, which make them unique among all others eukaryotic glycosyltransferases [22, 25]. This diversity, as well as the fact that *O*-glycosylation has no consensus sequence to occur, allows a fine tuned control of the initiation of this process in a specific cell or even in a specific protein [14, 26].

When the first step of the synthesis is concluded, a galactose (Gal) residue is transferred from an uridine diphosphate - galactosamine (UDP-Gal) donor to the GalNAc of the Tn antigen by a specific galactosyltransferase (β(1-3)-galactosyltransferase, C1Gal-T1 or T-synthase), yielding the most common core structure – core 1, also called T antigen or Thomsen-Friedenreich antigen (Galβ1-3Gal*N*Acα-*O*-Ser/Thr), as shown in **Figure 2** [14, 22, 25]. In 2009, Wang *et al.* proposed that the active state of T-synthase depends on the co-expression of an unusual molecular chaperone localized in the ER, *Cosmc* [27]. Apparently, this chaperone prevents the aggregation and proteasomal degradation of T-synthase, and is required for the export of the enzyme from the ER, leading to the formation of core 1 [27].

T antigen can be sialylated at *O*-3 position by a ST3Gal-sialyltransferase, yielding the sialyl-3-T antigen (S3T, Neu5Acα2-3Galβ1-3GalNAcα-*O*-Ser/Thr); this antigen can be further sialylated at *O*-6 position, by a ST6Gal-sialyltransferase, originating the di-sialyl-T antigen (diST, Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-*O*-Ser/Thr) (**Figure 2**) [14, 20]. Alternatively, the GalNAc residue of the T antigen may be sialylated at the *O*-6 position by a ST6GalNAc-sialyltransferase, yielding the sialyl-6-T antigen (S6T, Galβ1-3(Neu5Acα2-6) GalNAcα-*O*-Ser/Thr), which can then originate the diST antigen. Tn and T antigens, as well as their sialylated structures, block further elongation of the *O*-chain, and are generally designated simple mucin-type *O*-glycans [20, 28].

Core 1 may function as a precursor of other core structures (from core 2 to 8), by the addition of different monosaccharides, such as galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acids [19, 22]. However, cores 1-4 are the most common in humans – in fact, cores 5-8 have been characterized in tissues and appear to be formed

through direct modification of the nascent *O*-GalNAc glycan, but the enzymatic machinery underlying this process remains unclear [15, 20, 25].

Core 2 is synthesized in many epithelial (as intestinal mucosa) and hematopoietic tissues by the addition of a branching GlcNAcβ(1-6)-linked residue to core 1, by core 2 β(1-6) *N*-acetylglucosaminyltransferases (or C2Gn-T) (**Figure 2**) [14, 20].

Core 3 is formed by the addition of a GlcNAc residue by a core 3 β(1-3) *N*-acetylglucosaminetransferase (C3Gn-T3) to Tn antigen, and the subsequent addition of a GlcNAc residue onto core 3 by a C2Gn-T yields core 4, as represented in **Figure 2**. Thus, the prior synthesis of core 3 is required for the formation of core 4 [19, 20, 25].

The extension of core units provides a vast array of glycan structures, and is catalyzed by *N*-β3/4-acetylglucosaminyltransferases (β3/4 Gn-Ts) and/or β3/4-galactosyltransferases (β3/4 Gal-Ts), leading to the formation of side chains designated type-1 (Galβ1-3GlcNAc-R) and type-2 (Galβ1-4GlcNAc-R) chains [14, 19, 22]. These chains present a ubiquitous expression, and therefore are widely expressed among epithelial tissues. Type-1 and type-2 chains can be modified by the action of fucosyl and sialyltransferases, yielding ABO blood group determinants and/or Lewis blood group related antigens (Le^a, SLe^a, Le^x, SLe^x, Le^b and Le^y), which function as terminal structures by stopping chain elongation [14, 19, 22]. After *O*-glycosylation is concluded, the formed structures can undergo modifications, such as phosphorylation, sulfation and methylation, which constitutes an additional mean for their diversity [22].

O-GalNAc glycans are crucial structures for cells viability, once they play several and distinct roles in the organism, depending on the structure they present – this structural variability allows them to function as signaling, recognition and adhesion molecules [16, 17, 20].

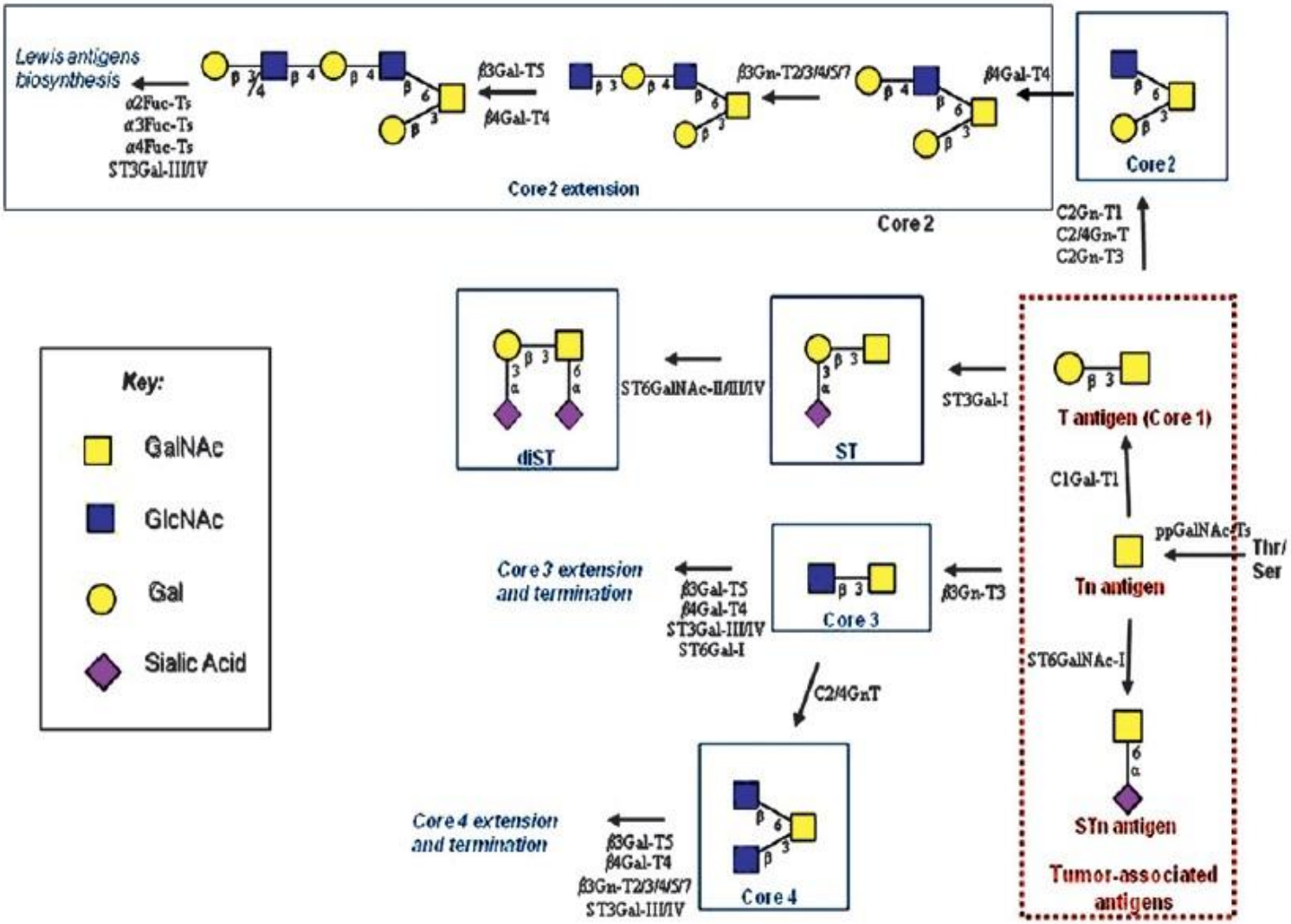


Figure 2 | Pathways of the biosynthesis of *O*-GalNAc glycans – synthesis of Tn and STn antigens and cores 1 to 4, which are the most common in humans (reprinted from [14]).

Enzymes: β(1-3)-galactosyltransferase (β3Gal-T); β(1-4)-galactosyltransferase (β4Gal-T); core 3 β(1-3) *N*-acetylglucosaminyltransferase (β3Gn-T); core 1 β(1-3)-galactosyltransferase (C1Gal-T1); core 2 β(1-6)-*N*-acetylglucosaminyltransferase (C2Gn-T); UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (ppGalNAc-T); α2,3-sialyltransferase (ST3Gal); GalNAc α2,6-sialyltransferase (ST6GalNAc).

Regulation of *O*-glycosylation

As *O*-GalNAc glycans are complex and widely diverse structures, their synthesis is tightly regulated. However, exactly mechanisms by which this regulation occurs remain to be clarified [20, 21]. The regulation of *O*-glycosylation seems to be dependent on the pattern of the enzymes involved in this process – substrate specificity, intracellular localization and level of relative activity are the main control factors of *O*-glycosylation [20, 21, 23].

Substrate specificity of glycosyltransferases and other enzymes involved in *O*-glycosylation allows a restriction of the number of possible *O*-GalNac glycans that can be formed, since these enzymes act in well-defined substrates [20, 25]. On the other hand, this specificity reduces the number of pathways by which *O*-glycans can be synthesized [20, 25]. For example, ST antigen can only be generated by the addition of a sialic acid residue to T antigen (core 1). The adding of a galactose residue to STn antigen does not yield the same structure, since the sialic acid present in STn antigen blocks the action of other glycosyltransferases, namely C1Gal-T1 [20].

Intracellular localization of glycosyltransferases is another important regulation factor of *O*-glycosylation, which is determined by the physical separation of enzymes within the Golgi compartments [25, 28]. These enzimes appear to be arranged in an assembly line in the Golgi apparatus – early acting glycosyltransferases occupy the *cis*-Golgi, intermediate acting enzymes are localized in the medial Golgi and terminal acting enzymes in the *trans*-Golgi, which allows a tight control of *O*-glycosylation [20, 28].

The third major control factor is the level of relative activity of glycosyltransferases, that consequently dictate the relative amounts of synthesized *O*-glycans – the activity of two competitive enzymes will determine the nature of the *O*-GalNAc glycan being synthesized, depending on which activity predominates [16, 25]. Besides this competition between glycosyltransferases, there is also the influence of other regulatory components, namely specific binding proteins required for the activation of glycosyltransferases [20]. Co-expression of *Cosmc* required for the activation of C1Gal-T1 is one example of this type of regulatory components [27].

Alterations of *O*-glycosylation in cancer

Abnormal protein *O*-glycosylation is considered a hallmark of malignant transformation [14, 20, 21]. Tumor-associated glycans can be found in the surface of cancer cells, and therefore are easily accessible to antibodies and lectins [29, 30]. Moreover, they are released to the peripheral circulation, either in secreted glycoproteins or by shedding from cell surfaces – these features allow them to be explored in various serological assays [20, 29]. Therefore, the presence of some tumor-associated glycans in serum is nowadays for post-surgical follow-up, to determine disease recurrence, progression and/or response to therapeutics [31].

Among the most common structural features associated with cancer are the altered expression of terminal structures, which includes loss of ABO determinants by secretor individuals and changes of Lewis antigenic patterns [20, 28], over-expression of mucins [32, 33], particularly MUC1, and incomplete *O*-glycosylation, yielding low molecular weight *O*-glycans [14, 20].

The first evidences of alterations in glycosylation patterns of tumor cells concerns loss of ABO blood group determinants in gastric cancer [34], and the correlation of the degree of A/B determinants deletion with invasion and metastatic spread was then described in lung [35] and head and neck [36] carcinomas. Thereafter, the relationship between expression of ABH antigens and survival of patients with non-small-cell carcinoma of the lung was also studied, and it was concluded that continued A determinant expression was correlated with a longest average survival, while A deletion was correlated with a reduced overall survival [37]. Nevertheless, correlation between B antigen expression and survival rate was not found [37]. Similar results were observed in bladder cancer patients with non-invasive and invasive carcinomas [38, 39].

Changes in Lewis antigenic patterns are related to over-sialylation of terminal structures, resulting in an over-expression of SLe^a and SLe^x antigens. The referred alterations in glycosylation stem from an aberrant expression of the genes encoding sialyl and fucosyltransferases, which increases the synthesis of these sialylated structures [14, 40]. The comprehension of the role of SLe^a and SLe^x in cancer begun in 1980s, when several studies demonstrated the expression of these antigens in cancer specimens, using monoclonal antibodies [41]. Later, in 1991, a study demonstrated the capability of SLe^a and SLe^x to function as selectin ligands for ELAM-1 (Endothelial Leukocyte Adhesion Molecule 1) in endothelials cells [42]. Subsequently, it was also discovered that this

mechanism allowed malignant cells to adhere to endothelium, facilitating invasion and metastasis [14]. Therefore, over-expression of SLe^a and SLe^x in different carcinomas has been demonstrated to be correlated with poor prognosis [17, 43].

Sialylated Lewis antigens can also be released to the bloodstream by malignant cells – thus, soluble forms of these antigens are also expressed in high amounts in the blood of many cancer patients [20, 21]. Studies based on the average survival of oncological patients with gastric carcinomas after surgery revealed that high levels of SLe^a and SLe^x correlated with lower survival [44, 45]. Based on these features, SLe^a and SLe^x are currently used in non-invasive assessment of tumor progression and metastatic spread [14, 17, 20].

The over-expression of mucins during neoplastic transformation, namely MUC1 mucin, has been widely documented [32, 33, 46]. MUC1 is a transmembrane glycoprotein with a large extracellular mucin-like domain, formed by 30-90 repeats of 20 homologous amino acids rich in *O*-glycosylation sites [47, 48]. Normally, MUC1 is expressed exclusively in the apical domain of epithelial cells; however, on carcinomas cells, the correct topology is lost and MUC1 is expressed over the entire cell surface, in abnormally large amounts [20, 33]. Since the extracellular domain of MUC1 is long, dense and relatively rigid, due to the abundance of *O*-glycan oligosaccharides, adhesion molecules present in cell surface, such as cadherins and integrins, become shielded. Thus, cell-cell and cell-extracellular matrix interactions decrease, creating an anti-adhesion effect [33, 49]. Consequently, MUC1 mucin has the capability to induce detach of a cell from the primary lesion, leading to invasion and metastasis. This process escapes from immune surveillance, because MUC1 inhibits the interaction between cytotoxic lymphocytes and the target cell and promotes apoptosis of lymphocytes, allowing the detach cell to survive in bloodstream or in distant organs [20, 49]. Moreover, the metastatic ability induced by MUC1 is also associated with the intracellular domain of the protein, which enters the nucleus and initiates the transcription of a set of genes responsible for tumor metastasis [50]. Over-expression of MUC1 is generally correlated with higher aggressiveness and metastatic capability of the tumor [33, 49].

Serological assays for MUC16 have also been shown the usefulness of this mucin in the prognosis of ovarian cancer, in which MUC16 is detected in 80% of the patients. Moreover, a correlation between increase/decrease of MUC16 expression and regression/progression of the disease has also been observed [14]. Similarly, serological

assays for MUC1 mucin in early stage breast cancer specimens have revealed that high levels of this glycoprotein are useful in the prognosis of the disease [40, 51].

The mechanisms underlying aberrant expression of mucins in malignant transformation are still poorly understood; nevertheless, it was suggested that they may arise from an up- or down-regulation of the genes encoding mucin proteins [13]. These genes may be deregulated in neoplasias originating from tissues where mucins are constitutively expressed, or these macromolecules may be ectopically expressed in cancers derived from tissues normally devoid of them [13].

Another common structural feature in cancer results from a premature stop of *O*-chain elongation, normally by sialylation. This event leads to *de novo* expression of a family of low molecular weight *O*-glycans, that includes Tn and T antigens and their sialylated counterparts [14, 20, 21].

High levels of ST and STn antigens have been observed in several carcinomas, namely gastric [18], colon [52], bladder [53], breast [40] and pancreatic [54], whereas low levels of expression are found in healthy tissues. Therefore, ST and STn antigens have been extensively studied in last decades and are widely assumed as pan-carcinoma biomarkers [18, 21, 55].

T antigen is also considered a pan-carcinoma antigen, once it is substantially over-expressed in several carcinomas, namely breast [40], bladder [53], colon [52], gastric [56] and prostate [57]. Nevertheless, the expression of T antigen is not limited to cancer – it is also expressed in normal tissues, although in low levels, since it undergoes further glycosylation [52, 56, 57]. In cancer tissues, the glycosylation process becomes incomplete, and the expression of T antigen increases significantly. The increment of T antigen expression is associated with a worse prognosis in cancer patients [58, 59].

The incomplete synthesis observed during malignancy can arise from diverse alterations in cancer cells. They commonly stem from altered regulation of sialyl and glycosyltransferases expression in cancer cells (up/downregulation) [14, 40, 53]. Namely, the level of expression of sialyltransferases in breast cancer has been studied as a biomarker for the follow-up of oncological patients – apparently, a higher expression of ST3Gal-III and ST6Gal-I in human breast tumors is correlated with poor prognosis [60]. At the same time, the activity of these enzimes may also be altered in cancer cells [18, 53]. Increase of sialyltransferases activity leads to preclude of *O*-glycosylation, due to the blocking effect of sialic acid on glycosyltransferases involved in chain extension [28]. Consequently, neoplastic cells express heavily sialylated and truncated *O*-GalNAc glycans,

namely ST and STn antigens, which have an increased ability to bind to adhesion molecules present in endothelial cells, such as selectins [18, 28, 53]. Therefore, these highly sialylated *O*-glycans have an enormous invasive potential and metastasis capability [18, 28].

Moreover, these alterations can also stem from a deregulation in the location of glycosyltransferases. Gill *et al.* (2010) [26] suggested a redistribution of ppGalNAc-Ts involved in *O*-glycosylation induced by the activation of Src kinase. This activation promotes a relocation of the enzymes from Golgi apparatus to the ER, allowing them to be more time in physical contact with potential substrates. Thus, occurrence of *O*-glycosylation is dramatically increased, resulting in an enhanced synthesis of truncated *O*-GalNAc glycans [26].

Another possible mechanism underlying the expression of cancer-associated *O*-glycans, suggested by Ju *et al.* (2011) [61], focus on a mutation on the molecular chaperone *Cosmc*, affecting the enzyme responsible for the synthesis of T antigen, T-synthase. According to Wang *et al.* (2009) [27], a functional T-synthase depends on the co-expression of a molecular chaperone, *Cosmc*. Ju *et al.* (2011) [61] also demonstrated that a mutation in the gene that encodes for *Cosmc* is responsible by the accumulation of cancer-associated precursors both in neoplastic lesions, including colon cancer and melanoma-derived cell lines, and in human cervical cancer specimens.

Overall, simple mucin-type *O*-GalNAc glycans are pan-carcinoma antigens, associated with malignant phenotypes such as increased invasive and metastatic potential – therefore, these structures correlate with poor prognosis [14, 18, 40].

STn – a pan-carcinoma antigen

STn detection among healthy tissues is heterogeneous [55, 62]. The expression of this antigen was found to be positive in normal salivary glands and in globlet and parietal gastric cells of upper digestive tract [55, 63, 64], but not in the esophagus [62]; conversely, STn can only be detected in colonic cells after removal of *O*-acetyl groups [65], and it was never found in normal liver and pancreas [62, 66]. In relation to the urogenital tract, STn was visible in some uterine and cervix cells, but not in the ovarian ones [55, 62]. Finally, it was also detected in some cells of normal lung [62] and breast [67] tissue. It is noteworthy that the authors report, overall, that the expression of STn antigen in normal tissues is rare

and/or low, when compared to cancer tissues. Moreover, these studies evidence an expression of STn restricted to secreting cells, which suggest that the spread expression of this antigen in healthy tissues relates to external fluids of the body [55].

STn antigen was also found to be over-expressed by pre-neoplastic lesions, such as gastric intestinal metaplasia, breast ductal hyperplasia and chronic ulcerative colitis [18, 55]. STn was also over-expressed in epithelial benign lesions in ovaries [68] and pancreas [69], two tissues devoid of this antigen in the healthy state. Of note, the expression of STn in pre-neoplastic pancreas occurred only in the last histological grade relative to benign tumor [69]. This suggests that STn over-expression occurs earlier in carcinogenesis in tissues that normally express this antigen, rather than in tissues normally devoid of STn [55].

In relation to cancer tissues, STn was reported to be neo- or over-expressed in more than 80% of human carcinomas, namely pancreatic [69], endometrial [70], breast [40], colon [71] and gastric [72]. Therefore, STn is considered a pan-carcinoma antigen and a good tumor marker of carcinogenesis [55]. In line with these observations, several *in vitro* and *in situ* studies have associated STn antigen with aggressive cell phenotypes [18, 40, 55]. It has been demonstrated that STn expression promotes major morphological alterations on the cell surface glycosylation profile, inducing or preventing the recognition by lectins. This process contribute to a cancer phenotype, decreasing cell-cell aggregation and increasing extracellular membrane adhesion, migration, invasion and metastasis [18, 55].

Kakeji *et al.* (1995) [73] have reported that an increased percentage of STn-positive cells was correlated with invasion in gastric cancer. In agreement with this study, Flucke *et al.* (2001) [74] reported a decreased overall survival of the patients with more than 35% of STn-positive cells in esophageal cancer, when compared to the low expressing group (<35% of STn-positive cells). Recently, Ozaki *et al.* (2012) [75] described that STn-positive gastric cancer cells have demonstrated higher intraperitoneal metastatic ability in comparison with STn-negative control in nude mice, resulting in depth of invasion and shorter survival. Moreover, Ogata *et al.* (1992) [76] have previously demonstrated that mucins bearing STn antigen are effective inhibitors of natural killer (NK) cells cytotoxicity. Thus, it was suggested that over-expression of STn in cancer specimens induces impairment in NK cell function of the immune system and, subsequently, that mucins expressing STn antigen allow cancer cells to escape from immune surveillance [76].

Given its biomarker value, STn was established as a tumor marker in serological assays (CA 72-4) [14, 55, 77]. This antigen is present in the bloodstream due to *O*-glycoprotein secretion or to cell shedding from tumors, which occurs only when the tumor reaches a critical mass. Therefore, the presence of STn in serum is usually detected in advanced tumors, and STn is considered a poor prognosis marker [55]. In fact, raised levels of serological STn have been associated with decreased overall survival of patients with gastric, ovarian and colorectal cancers [14, 55]. Overall, STn is considered a pan-carcinoma antigen, since it is expressed in the majority of human carcinomas, and it is associated with adverse outcome and decreased overall survival of the patients, as summarized in **Figure 3**.

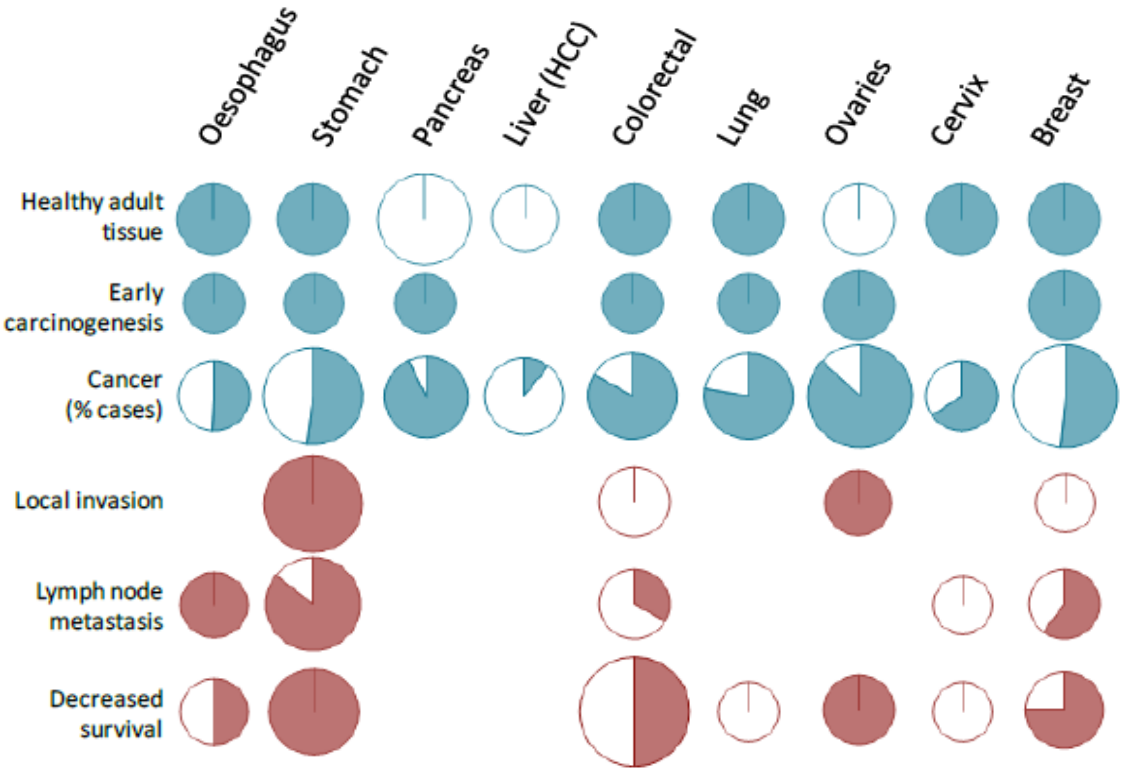


Figure 3 | Graphic overview of the expression of STn in cancer (adapted from [55])**.**

Top part: reports of positive (blue) or negative (white) expression in healthy, pre-malignant and cancer tissues. **Bottom part:** reports assessing the correlation between STn expression and clinical features of cancer, with a significant correlation found in pink and no correlation found in white. Size of circles is proportional to the number of published reports.

Obs.: The positive expression of STn in healthy adult tissue (first line) relates to a sparse or low expression, as described in the first paragraph of this section.

Given the cancer-associated nature of the STn antigen, a cancer vaccine named *Theratope*, comprehending a synthetic STn disaccharide coupled to a immunogenic carrier protein, keyhole limpet hemocyanin (KHL), was developed [55, 78]. Tests in animal models and phase II trials for breast and ovarian cancers have showed that the antigen is safe and produces a strong immune response against these tumors [78]. However, *Theratope* failed phase III trials for metastatic breast cancers, since it did not improved overall patient survival. In a recent review on the subject, Julien *et al.* (2012) [55] discussed that the failure of *Theratope* was related with the design of the study, namely that it disregarded the fact that only 20-30% of the patients with metastatic breast cancers expressed the STn antigen. In 2009, nonetheless, Julien *et al.* [79] investigated the efficacy of STn-carrying immunogens to inhibit tumor growth in a MUC1 transgenic mouse model. The authors reported that *Theratope* induced antibodies to STn that recognized the glycan carried in a number of glycoproteins. Moreover, a significant delay in tumor growth was observed in these mice, and the protection effect seemed to be dependent on STn being expressed by the tumor [79]. Altogether, the notion prevails that STn based therapeutics may constitute a strategy to control invasion and metastisis, and consequently poor outcomes. The fact that it is a cell-surface antigen that may be more easily accessible to antibodies and/or other ligands also offers potential in the context of guided therapeutics.

Abnormal *O*-glycosylation in bladder cancer

The first reports of alterations in glycosylation of bladder tumors have been presented over 40 years ago and relate to the loss of ABO blood group determinants in advanced stage carcinomas of secretor individuals (the *Se* – secretor – locus dictates the capability of an individual to express soluble mucins carrying blood group antigens in saliva and other tissues). Subsequent studies have shown that malignant transformations in the bladder are accompanied by changes in Lewis antigens pattern and over-expression of simple mucin type *O*-GalNAc glycans. **Table 1** summarizes the literature on the subject in healthy, pre-neoplastic and neoplastic urothelium and its correlation with clinicopathological features. The studies focused on the expression of STn, the core antigen of this thesis, in the context of bladder cancer will be discussed further forward, in the subtopic **2.3.1.**.

Six studies have described the loss of ABO blood group antigens in healthy and neoplastic urothelium, as documented in **Table 1**. Nevertheless, this event is more

pronounced in the neoplastic urothelium (44-100%) when compared with the healthy urothelium (0-24%). Thorpe *et al*. (1983) [80] and Summers *et al*. (1983) [81] have correlated the loss of ABO antigens with the invasive potential of the tumor; moreover, Limas *et al*. (1985) [82] have also associated this event with higher grade bladder cancer.

Altered Lewis antigenic profile has been reported in pre-neoplastic and neoplastic urothelium (**Table 1**). Namely, several authors have studied the expression of Le^a in bladder tumors. According to Cordon-Cardo *et al.* (1988) [38], there were no alterations in Le^a expression patterns with malignant transformations in the bladder. Conversely, Limas *et al.* (1985) [82] reported significantly lower expression of this antigen in healthy urothelium (6%), when compared to invasive tumors of the bladder (35%). In agreement with this study, Juhl *et al.* (1986) [39] reported a high expression of Le^a antigen in invasive bladder carcinomas (93%). Both these studies were performed using a boarder patient cohort (n>35) than the one used by Cordon Cardo *et al.* (n=19), and strongly suggest that the expression of Le^a is associated with a malignant phenotype of bladder tumor [39, 82].

The sialylated form of Le^a, the SLe^a antigen, has been observed in bladder dysplasia, CIS, non-invasive and invasive carcinomas of the bladder [83]. Nevertheless, no correlation was found with invasive or metastatic potential was not established.

Three studies referred in **Table 1** have focused on the expression pattern of Le^x antigen, and revealed a high percentage of bladder cancer patients expressing altered levels of Le^x (78-100%). Specifically, Cordon-Cardo *et al.* (1988) [38] have compared the expression pattern of this antigen in healthy urothelium with invasive carcinomas and CIS. The study revealed that none of the healthy urohelium specimens expressed Le^x; conversely, the antigen was observed in invasive carcinomas of the bladder (100%) and CIS (79%). Furthermore, the authors proposed that Le^x expression could be a reliable indicator of malignant transformation in bladder urothelium [38].

Its sialylated form, SLe^x expression has also been studied in bladder urothelium, as demonstrated in **Table 1**. According to Numahata *et al.* (2002) [84], 70% of the invasive carcinomas expressed SLe^x expression. These findings were corroborated by Kajiwara *et al.* (2005) [85] that observed altered SLe^x patterns in all of the invasive carcinomas specimens studied. Both studies referred altered SLe^x expression as a predictor of invasive potential and metastatic outcome.

Increased levels of truncated *O*-GalNAc glycans have also been observed in bladder cancer (**Table 1**). Yokoyama *et al.* (1988) [86] have compared the spontaneous expression

of T antigen (48%) with it expression after neuraminidase treatment (cryptic-T antigen (18%)), in bladder cancer specimens of various stages. The authors revealed that 70% of the T-positive cases presented recurrence, while only 11% of the cryptic-T-positive did. Similarly, Summers *et al.* (1983) [81] reported that those patients who did not spontaneously expressed the T antigen (54%), did not suffer invasive recurrences; conversely, the patients that expressed spontaneous T antigen (38%) suffered subsequent invasive recurrences.

Tn (79%), STn (3%), T (29%) and cryptic-T (53%) antigens were evaluated by Langkilde *et al.* (1992) [12] in patients with initially non-invasive carcinomas who experienced different courses of the disease. No association was found between Tn and STn antigens and tumor progression to invasion. Nevertheless, 70% of the patients that expressed T antigen and 39% of those who expressed cryptic-T antigen experienced invasive recurrence, while the other patients did not [12].

Furthermore, Limas *et al.* (1986) [87] demonstrated a crescent expression of T antigen through healthy (0%), pre-neoplastic (14%) and neoplastic (11-65%) urothelium. Cryptic-T antigen showed a homogeneous expression among all the specimens evaluated (78-100%). According to the authors, the spontaneous expression of T antigen is associated with aggressiveness of the tumor; moreover, in invasive carcinomas of the bladder, this expression correlates with a greater metastatic potential.

Altogether, these findings suggest that bladder cancer patients who spontaneously express T antigen present invasive recurrences more frequently than those who do not express this antigen; furthermore, T antigen expression seems to correlate with metastatic potential and aggressiveness of the tumor [12, 81, 86].

The studies presented so far suggest that both pre-neoplastic lesions and bladder tumors express altered glycosylation patterns. Some studies also point out that changes in cell glycosylation patterns are generally associated with tumor grade, invasive disease and metastasis – therefore, they are correlated with poor prognosis [81, 86]. Thus, targeting these antigens may allow determining the malignant potential of the tumor and controlling the disease. Nevertheless, there studies have been performed in small and heterogeneous patient cohorts using different antibodies. Therefore, a careful interpretation of the results should be conducted, since several antibodies have shown to have affinity for similar structurally-related glycosylated structures [88]. Different methodologies have also been used, which may contribute to biased interpretations.

Table 1 | Literature review on the expression of tumor-associated glycans in healthy, pre-neoplastic and neoplastic urothelium.

	n	Positive cases (%)									Relates with	Technique	References
		Tn	STn	T	Cryptic-T	Loss of ABO*	Le^{a*}	SLe^{a*}	Le^{x*}	SLe^{x*}			
Healthy urothelium	19	-	-	-	-	16	63	-	0	-	-	IHQ	[38]
	21	-	-	-	-	24	-	-	-	-	-	IF	[80]
	35	-	-	-	-	0	6	-	-	-	-	RCA	[82]
	24	-	-	-	-	8	-	-	-	-	-	IHQ	[89]
	30	-	-	0	80	-	-	-	-	-	-	IHQ	[87]
	6	-	0	-	-	-	-	-	-	-	-	IHQ	[11]
Pre-neoplasia													
Dysplasia[1]	2	-	-	-	-	-	-	50	-	-	-	EIA	[83]
Atypia	7	-	-	14	100	-	-	-	-	-	invasion/metastasis	IHQ	[87]
Neoplastic urothelium													
	39	-	-	38	54	54	-	-	-	-	invasion	IHQ	[81]
NMIBC	4	-	-	-	-	100	-	-	-	-	invasion	IF	[80]
(except CIS)	27	-	-	11	78	-	-	-	-	-	invasion/metastasis	IHQ	[87]
	42	-	-	-	-	-	-	64	-	-	-	EIA	[83]
	34	79	3	29	53	-	-	-	-	-	invasion	IHQ	[12]
	45	-	47	-	-	-	-	-	-	-	proliferation	IHQ	[11]

[1] Evaluated indirectly in exfoliated urine cells
* May also be found as terminal structures of *N*-glycans and lipids

	n	Positive cases (%)									Relates with	Technique	References
		Tn	STn	T	Cryptic-T	Loss of ABO*	Le^{a*}	SLe^{a*}	Le^{x*}	SLe^{x*}			
MIBC	44	-	-	-	-	-	-	-	-	70	invasion/metastasis	IHQ	[84]
	33	-	-	-	-	-	-	76	-	-	-	EIA	[83]
	19	-	-	-	-	-	53		100	-	-	IHQ	[38]
	1	-	-	-	-	100	-	-	-	-	invasion	IF	[80]
	48	-	-	-	-	44	35	-	-	-	grade/invasion	RCA	[82]
	17	-	-	65	88	-	-	-	-	-	invasion/metastasis	IHQ	[87]
	52	-	-	-	-	-	-	-	-	100	invasion/recurrence	IHQ	[85]
	85	-	-	-	-	-	93	-	-	-	invasion/recurrence	IHQ	[90]
	93	-	-	-	-	44	-	-	-	-	-	IHQ	[39]
	19	-	74	-	-	-	-	-	-	-	proliferation	IHQ	[11]
CIS	1	-	-	-	-	-	-	100	-	-	-	EIA	[83]
	14	-	-	-	-	50	64		79	-	-	IHQ	[38]
	5	-	-	20	100	-	-	-	-	-	invasion/metastasis	IHQ	[87]
	6[2]	-	-	-	-	-	-	-	100	-	-	IHQ	[91]
	5	-	20	-	-	-	-	-	-	-	proliferation	IHQ	[11]
Not discriminated	83	-	-	48	18	-	-	-	-	-	grade/recurrence	IF	[86]
	32	-	-	-	-	-	-	-	81	-	-	IHQ	[92]
	78	-	-	-	-	-	-	-	78	-	-	IHQ	[91]

– Not evaluated

[2] Evaluated the urinary levels
* May also be found as terminal structures of *N*-glycans and lipids

Technique's Abbreviation:

IHQ – Immunohistochemistry
IF – Immunofluorescence
RCA – Red Cell Adherence Test
EIA – Enzyme Immuno Assay

The STn antigen in bladder cancer

Despite the cancer-associated nature of the STn antigens, few studies have been presented for bladder cancer (**Table 1**). The first study by Langkilde *et al.* (1992) [12] assessed this antigen on a series of transitional cell carcinomas (currently classified as high-grade urothelial cell carcinomas according to WHO guidelines), and in a control group, comprehending normal mucosal specimens of patients with non-malignant bladder urologic diseases. The authors reported that STn was not expressed by the control group and showed a very restricted pattern of expression in bladder tumors. Moreover, no association with recurrence and progression were observed [12].

In vitro studies performed by Bergeron *et al.* (1996) [13] showed that mucins MUC1, MUC2 and MAUB (mucin antigen of the urinary bladder) isolated from bladder cancer cell lines carried the STn antigen. Nevertheless, that expression was not found in tumors.

Recently, Ferreira *et al.* (2013) [11] addressed the expression of STn in 6 necropsies of normal urothelium, as well as in 69 bladder cancer patients. STn antigen was absent from the healthy urothelium, showing the STn tumor-associated expression. The authors also reported that approximately 70% of high-grade (HG) bladder tumors over-expressed STn. Conversely, only less than 25% of low-grade (LG) tumors over-expressed this antigen – altogether, the obtained results suggest that the expression of STn is associated with advanced stage bladder tumors, known for an aggressive phenotype. Moreover, Ki-67 antigen, a proliferation tumor marker, was also assessed in 12 LG and 12 HG tumors from the initial series. Only 8% of LG bladder tumors over-expressed Ki-67, as opposed to 75% of HG tumors – overall, these findings suggest that the expression of STn is a characteristic of proliferating tumors [11]. *In vitro* studies have further demonstrated that STn expression enhanced the invasion capability of bladder cancer cells.

Despite these observations, little information is available about the biological and clinical significance associated with STn expression in bladder cancer. Namely, doubts persist about its association with invasion and metastasis, which is of prime importance to encourage the development of therapeutics targeting this antigen.

Chapter II | Aims and scopes

Aims and scopes

The main therapeutic concerns in bladder cancer currently includes the management of NMIBC at a high-risk or recurrence with progression, that do not respond or show intolerance to BCG immunotherapy, and MIBC [7, 10]. These patients are conservatively treated by partial or radical cystectomy with neo- and post-surgical chemotherapy to decrease the risk of metastasis. However, over 50% of the patients succumb within five years [5, 11]. Due to the limited therapeutic options, the identification of a specific biomarker associated with invasion and metastasis could encourage the development of new treatments.

The modification of cells glycosylation patterns is a recognized hallmark of cancer [14, 20, 21] and the STn antigen, in particular, presents potential to target malignant cells in invasive bladder tumors. It is known that STn contributes to avoid metastatic cell elimination in the blood stream by preventing immune recognition [76], modulates the malignant phenotype [18] and enhances the metastatic ability of cancer cells [75]. Furthermore, this antigen has already been suggested to be over-expressed by 70% of high-grade NMIBC and MIBC cases and therefore associated with tumor aggressiveness [11]. However, this study was conducted in a rather small and heterogeneous series (n=69) comprehending only few MIBC cases. The present work aims to determine the association of STn with invasion as well as lymph node and distant metastasis. The second part of the work focuses on the validation of a STn-expressing bladder cancer xenograft model to support drug testing. The generated information is regarded of primary importance to expand the knowledge about the clinical relevance of the STn antigen in bladder cancer and create the rationale for a STn-based therapy.

Chapter III | Material and methods

Overview

The first part of the project devotes to verify the association of the STn antigen with invasion in a series of 96 primary bladder tumors including different stages of the disease (Ta, T1, T2, T3 and T4). This series will also be characterized according to the degree of proliferation, based on Ki-67 expression. Moreover, STn expression will be evaluated in a a smaller and independent series of 21 bladder tumors isolated by radical cystectomy, presenting lymph node (17 cases) and distant metastasis (6 cases). This will allow determining the association of the STn antigen with metastasis. Finally, glycoproteins will be extracted from bladder tumors and metastasis to disclose the STn-expressing glycoprotein profiles.

The second part of the thesis devotes to the validation of a STn-expressing bladder cancer xenograft model in nude mice, by immunohistochemistry and Western blot. Such model is regarded of primary importance to identify drugs and treatment regimens that would better serve patients with STn-positive MIBC, as well as a platform to identify markers of tumor response and resistance to chemotherapic agents.

An overview on the analytical strategy adopted is presented in **Figure 4**.

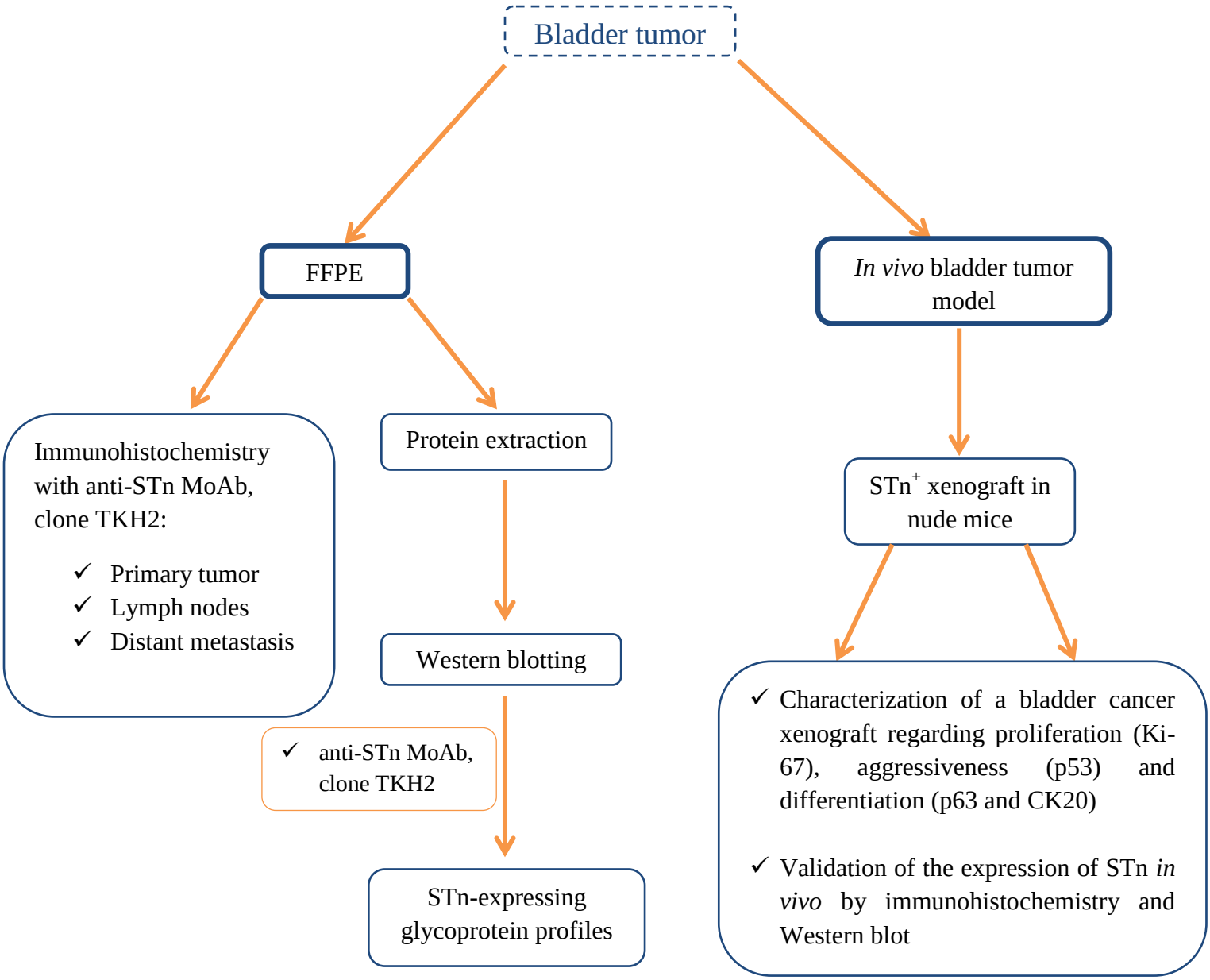


Figure 4 | Overview on the analytical approach. FFPE – formalin fixed paraffin embedded; MoAb – monoclonal antibody; CK20 – cytokeratine 20.

Chapter IV | Sialyl-Tn over-expression is associated with invasion and metastasis in bladder cancer

Sialyl-Tn over-expression is associated with invasion and metastasis in bladder cancer

Abstract

Introduction: Approximately 50% of muscle invasive bladder cancers (MIBC) develop metastasis within 5 years after surgery, despite being subjected to neo- and post-surgery chemotherapy regimes. Thus, specific biomarkers to target aggressive cell phenotypes and direct molecular-based therapy are warranted. Recently, evidences have been presented that over 70% of advanced stage bladder cancers expressed the cell-surface tumor-associated carbohydrate antigen sialyl-Tn, which may be used to target aggressive bladder cancer cells. **Objective:** Determine the association of the STn antigen with invasion and metastasis and therefore contribute to determine the clinical relevance of this antigen in bladder cancer. **Methods:** The study included 96 patients with bladder cancer of different stages. Moreover, 21 cases with lymph node metastasis, distant metastasis or both were also included, as well as the correspondent primary tumors. All cases were screened for STn expression by immunohistochemistry. The series of 96 tumors was further characterized in relation to their degree of proliferation based on Ki-67 expression. The STn glycoprofilling of primary tumors and metastasized ganglia was evaluated by Western blot. **Results:** All STn-positive tumors presented a proliferative phenotype; however, this antigen was mainly absent from proliferation areas of the tumor. STn expression was found associated with invasion of the basal, muscle and fat layers and STn expressing cells were frequently observed in invasion fronts. STn expression was also present in lymph node and distant metastasis. The STn antigen was mainly observed in high molecular weight glycoproteins (>250 kDa) and what appears to be its proteolysis products between 25 and 10 kDa. These low molecular weight species predominate in lymph node mestastasis samples that also do not present high molecular weight proteins, suggesting a molecular signature associated with metastasis. **Conclusions:** The STn antigen is associated with tumor invasion and can be observed in lymph node and distant metastasis, offering potential for the development of new therapies against aggressive bladder cancer. Studies should be conducted to determine the nature of the STn-expressing glycoproteins and disclosing the possibility of a molecular signature associated with metastasis and its biological significance.

Keywords: invasive bladder cancer, metastasis, sialyl-Tn, glycosylation

Introduction

Bladder cancer is the fifth most common cancer in Western society [1–3] and presents the highest recurrence rate among solid tumours and poor prognosis when the tumour invades the *muscularis propria* [2, 4].

Approximately 10% of newly diagnosed cases are muscle invasive bladder cancers (MIBC). Furthermore, approximately 50% of non-muscle invasive bladder tumors (NMIBC) comprehending high-grade papillary tumours, carcinoma *in situ* and multifocal and/or recurrent lesions progress to MIBC if left untreated. Although BCG immunotherapy has improved the management of these patients, 10-20% of the patients still progress after treatment [10].

Muscle invasive bladder cancers (MIBC) are submitted to pre- and post-cystectomy adjuvant chemotherapy; despite this, approximately 50% of these cases develop metastasis within 5 years after surgery [5, 11]. Thus, at the moment, there is a lack of specific biomarkers to target aggressive cell phenotypes and direct molecular-based therapy, which may be used to avoid preventive cystectomy and/or reduce the chance of poor outcome [3]. In this context, recent studies have demonstrated that cell-surface cancer-associated carbohydrates antigens can be used for selective target delivery.

It has been long described that aggressive urothelium cancers in individuals of the secretor phenotype lose the ability to express cell-surface ABO(H) blood group determinants, reflecting profound alterations in glycosylation pathways [20, 28]. However, little is known about the glycosylated structures formed thereafter. Following these observations, we recently described that approximately 70% of high-grade bladder tumours expressed the sialyl-Tn (STn) that results from a premature stop in the *O*-glycosylation of cell-surface proteins [11]. Conversely, less than 25% of the low-grade cases over-expressed the STn antigen. Studies *in vitro* have further demonstrated that STn expression promoted the motility and invasive potential of bladder cancer cells [11]. Altogether, these findings suggested that STn expression may be associated with tumour invasion and metastasis; however, this remains to be verified. Herein, we devoted to determine the association of STn with invasion and metastasis in bladder tumours. This will help to determine the potential of this epitope in the context of novel therapeutics for bladder cancer.

Materials and Methods

Population

Ninety six bladder cancer patients treated at the Portuguese Institute of Oncology (IPO-Porto) between 1998 and 2010 were enrolled in this retrospective study. This comprehends 82 males and 14 females ranging in age from 38 to 92 years (median of 69.5 years). Forty seven bladder tumors were histologically classified as superficial non-muscle invasive bladder cancer (NMIBC; pTa and pT1) and 49 as muscle-invasive tumors (MIBC; pT2-pT4); moreover, 16 cases were classified as low-grade and 80 as high-grade, according to the 2004 WHO stage and grading criteria, respectively. Twenty-one primary bladder tumors showing lymph node metastasis (17 cases) and distant metastasis (6 cases) were also involved in this study. The average follow up time period was 31.5 months (0 – 79 months). Cystectomy was performed in 64 (66.7%) patients while the other 32 (33.3%) were submitted to transurethral resection.

Expression of STn in bladder tumours

Formalin fixed paraffin embedded (FFPE) tissue sections were screened for STn and Ki-67 by immunohistochemistry, using the avidin/biotin peroxidase method, as described by Ferreira *et al.* (2013) [11]. Briefly, 3 μm sections were deparaffinised with xylene, rehydrated with graded ethanol series, microwaved for 15 minutes in boiling citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 minutes. The expression of STn was then evaluated using anti-STn mouse monoclonal antibody, clone TKH2 [93], that identifies both single and clustered STn residues [65], whereas Ki-67 was evaluated using monoclonal mouse anti-human Ki-67 antibody, clone MIB-1 (Dako). After blockage with BSA (5% in PBS), the antigens were identified with VectastainEliteABC peroxidase kit (Vector Laboratories) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the slides were counterstained with haematoxylin for 1 minute. Positive and negative control sections of intestinal metaplasia were tested in parallel. The negative control sections were performed by adding BSA (5% in PBS) devoid of primary antibody. STn positive tissues were also treated with a α-neuraminidase from *Clostridium perfringens* (Sigma

Aldrich), as previously described by Marcos *et al.* (2011) [94], in order to remove the sialic acid. The desialyated samples were thereafter screened for STn.

The STn and Ki-67 expression were assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached. Tumours were classified as STn-positive when immunoreactivity with anti-STn TKH2 antibody was observed in more than 5% of tumor cells. Tumours were classified as proliferative whenever Ki-67 expression was higher than 18%, as described by Santos *et al.* (2003) [95].

Protein extraction and Western blot

Proteins were extracted from FFPE tissues using the Qproteome FFPE tissue kit (Qiagen). The amount of protein in each extract was estimated with DC protein assay kit (BioRad). Protein lysates were then separated by 4–16% gradient SDS–PAGE under reducing conditions and transferred onto 0.45 μm nitrocellulose membranes (GE Healthcare). Membranes were blocked with 1% carbohydrate depleted carbo-free solution (Vector Lab) for 1 hour at room temperature, incubated overnight at 4ºC with anti-STn TKH2 monoclonal antibody in culture supernatant, washed with TBS-T for 30 minutes, and finally incubated for 45 minutes with goat anti-mouse IgG1 heavy chain horseradish peroxidase conjugate (Abcam; 1:35,000 in TBS). After washing, the bound antibodies were revealed by chemiluminescence using the ECL Prime Kit (BioRad). Beta-actin expression determined with rabbit anti-beta Actin polyclonal antibody (Abcam) was used to control protein loads.

Statistical analysis

Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0). Chi-square analysis was used to compare categorical variables.

Results

A series of 96 primary bladder tumors was screened for the expression of the STn antigen, by immunohistochemistry, to determine associations with invasion. The tumors were further characterized in relation to their degree of proliferation based on Ki-67 expression. The association of STn with metastasis was assessed in a small series of 21 primary bladder tumors showing metastized ganglia (n=17) and/or distant metastasis (n=6). The STn glycoprofilling of primary tumors and metastasized ganglia was also evaluated by Western blot.

Association of STn expression with invasion and proliferation

All of the STn expressing cells presented membrane and cytoplasmic staining (**Figure 7**), in accordance with previous reports from Ferreira *et al.* (2013) [11]. STn expression was mainly observed in highly undifferentiated cells characterized by high nuclear to cytoplasmatic ratio. Control tissue sections, subjected to treatment with an α-neuraminidase that removes sialic acids, failed to present reactivity with monoclonal antibody TKH2, thereby confirming the specificity of the immunostaining.

As can be observed in **Figure 5**, STn was mainly expressed by tumors showing invasion of the *lamina* (T1; 60%) and the *muscularis propria* (≥T2; approximately 60-90%); conversely, the percentage of positive Ta was lower than 30% ($p<0.001$). The presence of the STn antigen was statistically associated with MIBC when compared to NMIBC ($p=0.001$), represented in **Figure 6**. Furthermore, STn expression was also statistically associated with high-grade tumours ($p=0.012$; data not shown).

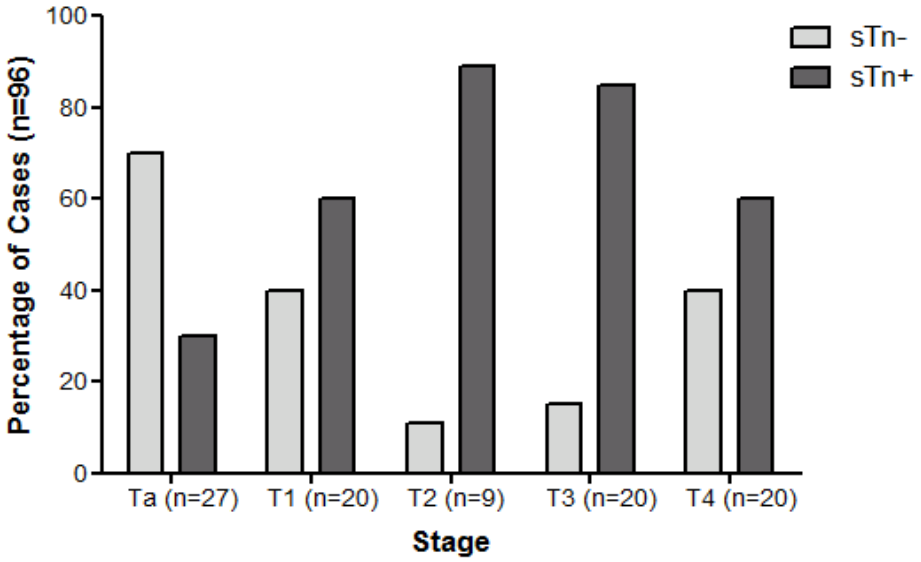


Figure 5 | Association between STn expression and tumor stage. STn is mainly expressed by tumors showing invasion of the *lamina* (T1, n=20) and the *muscularis propria* (≥T2, n=49). Conversely, the percentage of STn-positive Ta (n=27) is lower than 30%. $p<0.001$ (Chi-square test).

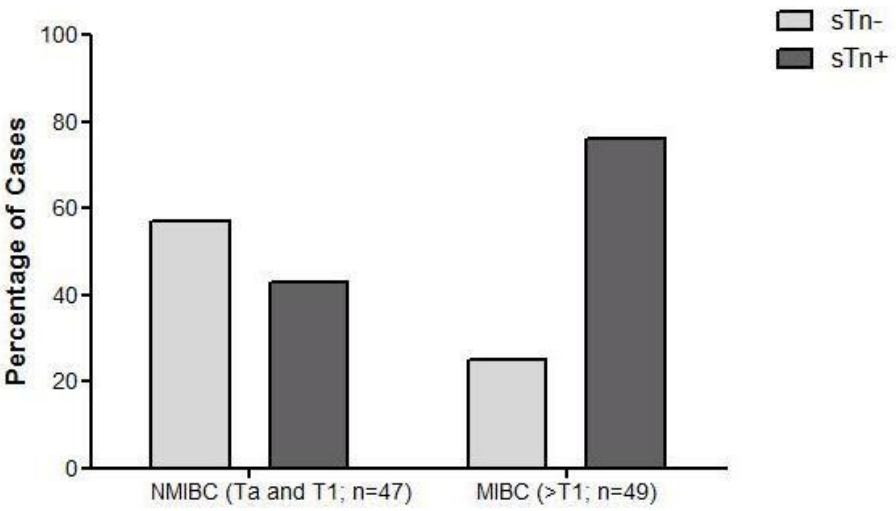


Figure 6 | Association between STn expression and NMIBC and MIBC. About 80% of MIBC (n=49) cases were STn-positive, contrasting with less than 60% of NMIBC (n=47) cases. $p=0.001$ (Chi-square test).

Adding to these statistical associations with invasion, STn-positive cells could be observed in invasive areas of the tumor, reinforcing a possible role of this antigen in invasion (**Figure 7**).

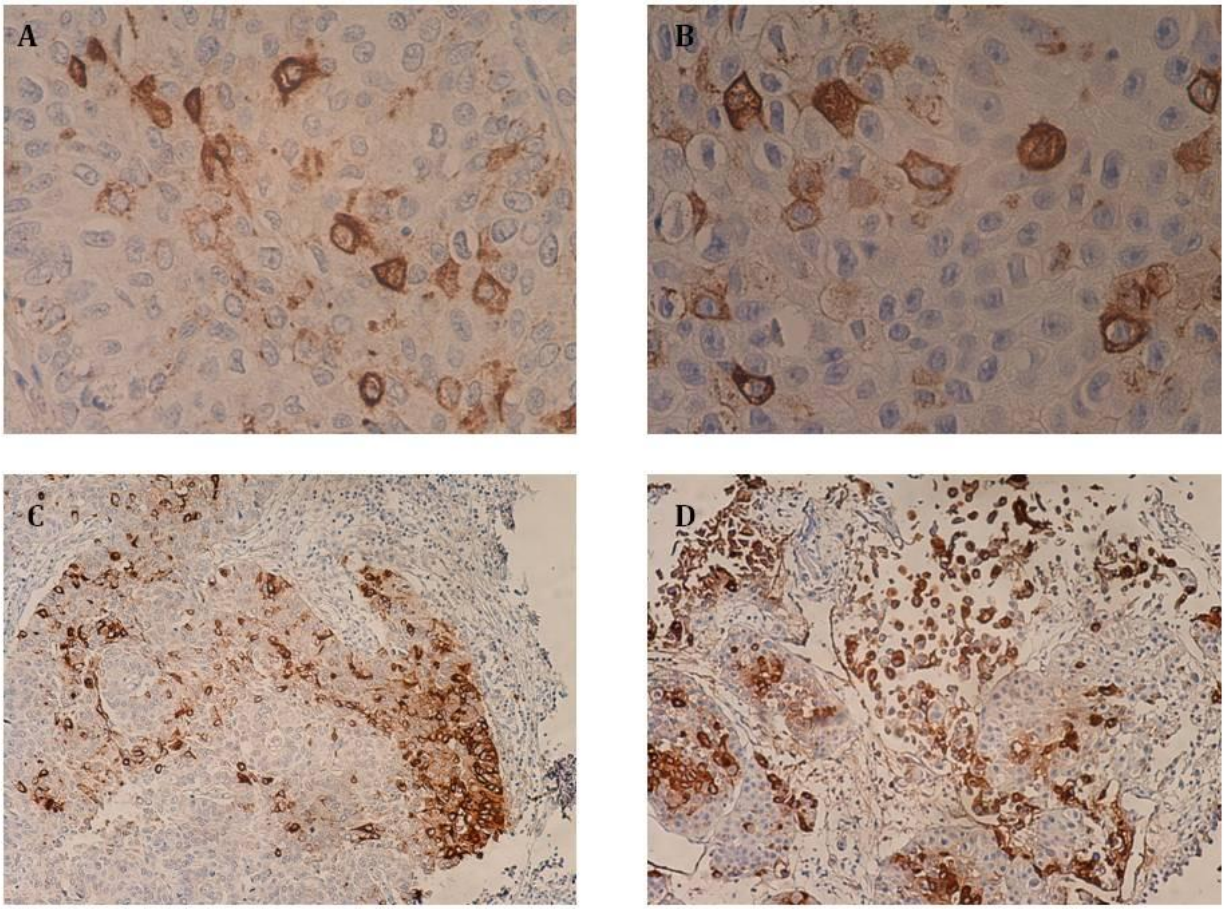


Figure 7 | Expression of STn in bladder tumors. A) and B) Magnification showing undifferentiated tumor cells (high nuclear/cytoplasmatic ratio), with membrane and cytoplasmatic STn^+ staining; **C)** High-grade papillary tumor showing invasion of the *lamina propria* and extensive STn positivity; **D)** MIBC highlighting extensive STn expression including in invaded areas of the muscle layer.

It is also noteworthy that all of the STn-positive bladder tumors studied (approximately 60%) presented a proliferative phenotype. However, as shown by **Figure 8**, STn was mainly expressed in non-proliferative tumor areas, which reinforced previous observations by Ferreira *et al.* (2013) [11].

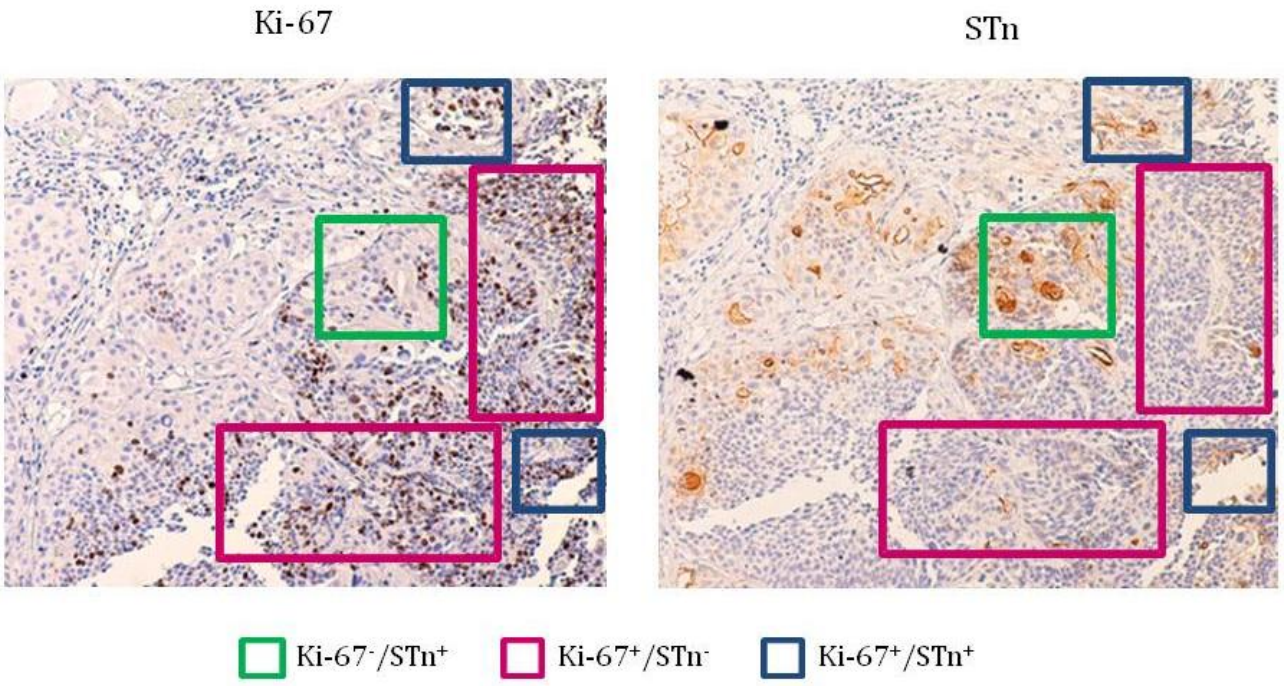


Figure 8 | Expression of STn and Ki-67 in bladder tumors. The marked areas highlight that STn is mainly observed in non-proliferative areas of proliferative tumors, with some overlapping zones.

Association of STn expression with lymph node and distant metastasis

The association of STn with metastasis was screened in a small series including 21 primary bladder tumors, 17 of each with available metastized ganglia and 6 distant metastasis. Approximately 88% of the metastized ganglia (15/17) and all of the distant metastasis (6/6) screened for this antigen were STn-positive (**Figure 9**). Furthermore, the STn antigen was over-expressed in 93% of the tumors with lymph node metastasis (14/15), as also shown by **Figure 9**. In agreement, the two metastized ganglia which were STn-negative also presented primary tumors STn-negative. Therefore, a high STn expression in the tumor is associated with lymph node metastasis (p=0.029). As illustrated by **Figure 9, i**t is also noteworthy that all of the tumors presenting distant metastasis (6/6) over-expressed STn. **Figure 10** highlights the extensive STn⁺ staining in both lymph node and distant metastasis. Furthermore, tumor cells with membrane and cytoplasmatic STn expression are also evidenced in **Figure 10**. Altogether, these data suggest the association of the STn antigen with the metastization process.

	MT	LNM	DM
1	+	+	n/a
2	+	+	+
3	+	n/a	+
4	+	n/a	+
5	+	n/a	+
6	+	+	n/a
7	+	+	n/a
8	+	+	n/a
9	+	n/a	+
10	+	+	n/a
11	-	-	n/a
12	-	-	n/a
13	+	+	n/a
14	+	+	n/a
15	+	+	n/a
16	+	+	n/a
17	-	+	n/a
18	+	+	n/a
19	+	+	n/a
20	+	+	n/a
21	+	+	+

Legenda:

NMIBC (T1)

MIBC

n/a Not available

Figure 9 | Expression pattern of STn in primary bladder tumors (MT, main tumor) and the correspondent lymph node (LNM) and/or distant metastasis (DM). The graphical matrix highlights an over-expression of the STn antigen in the majority of the metastized ganglia (88%) and in all cases of distant metastasis (100%), suggesting an association between STn and the metastatic event.

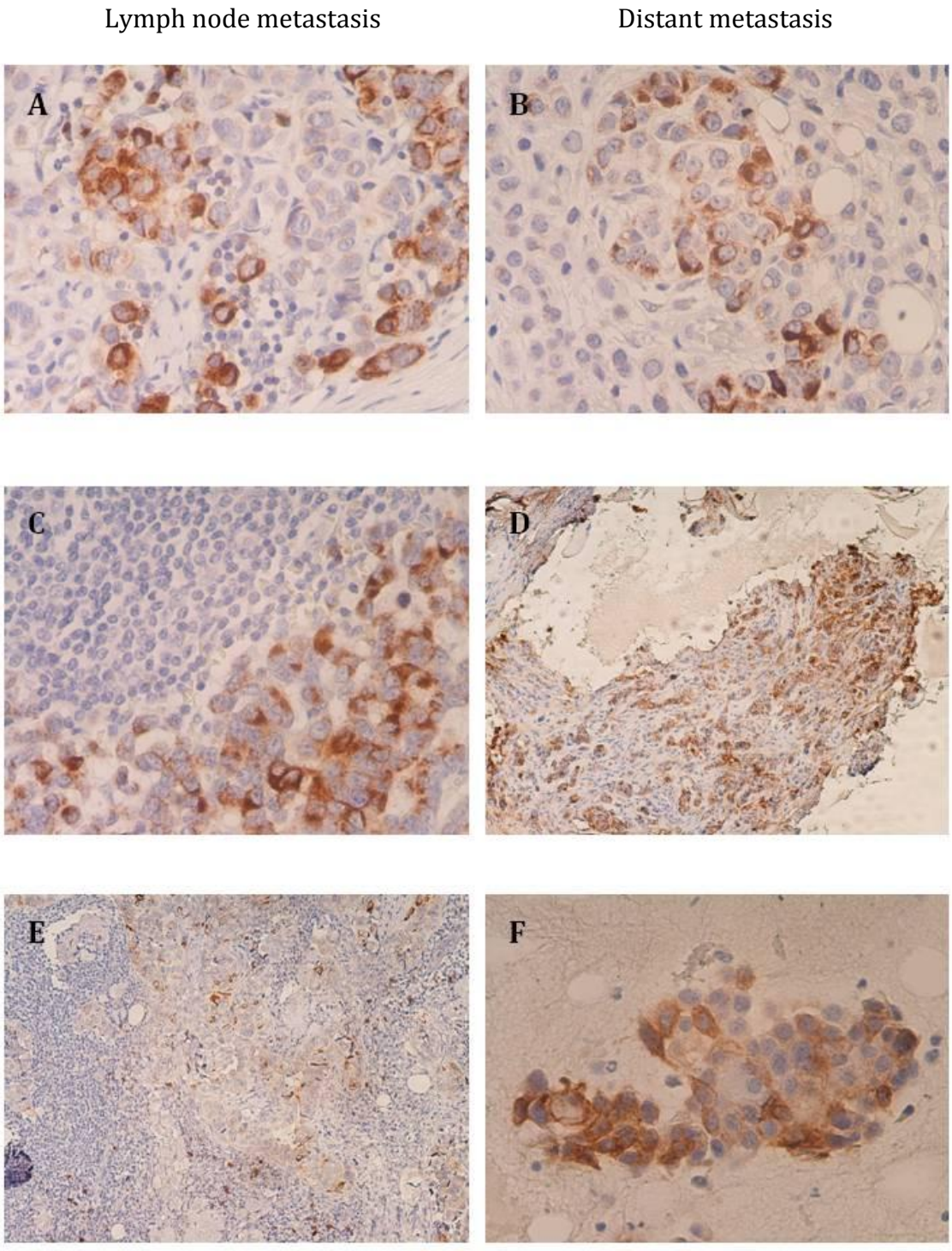


Figure 10 | Expression of STn in lymph node and distant metastasis. A) and B) Magnification showing tumor cells with membrane and cytoplasmatic STn⁺ staining. **C)** Lymph node metastasis evidencing localized STn expression. **D)** Distant metastasis showing an intense STn⁺ staining. **E)** Lymph node metastasis highlighting locally extensive STn expression. **F)** Intense STn⁺ staining in a biopsy of a distant metastasis.

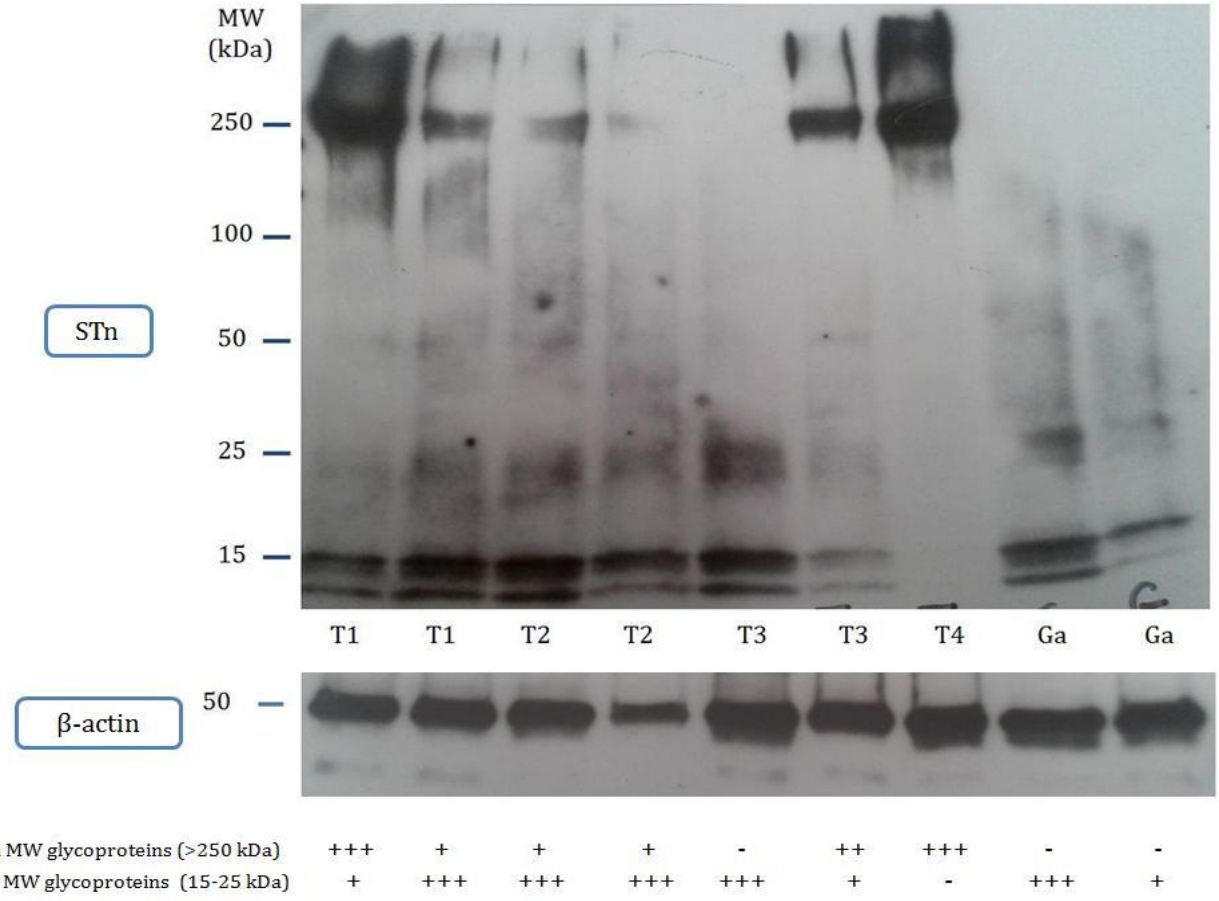
STn glycoprofiling

Proteins were extracted from nine STn positive bladder tumors (T1, n=2; T2, n=3; T3, n=3; T4, n=1) and two ganglia presenting metastasis that were isolated and blotted with an anti-STn monoclonal antibody, in an attempt to characterize the protein expression pattern for this antigen.

Figure 11 demonstrates the staining pattern for 7 out of the 9 primary bladder tumors studied, as well as the two ganglia. A band was visible at 250 kDa in almost all of the primary tumors (including the two tumors not shown in **Figure 11**), especially in the first T1 and T4 cases. This band is concordant to the presence of mucins (MUC), possibly MUC1, a high-molecular weight transmembrane protein previously reported to be one of the main proteins expressing the STn antigen [18, 66, 75].

The western blot also presented three low-molecular weight bands at 25, 15 and 10 kDa, with the exception of the proteins extracted from the T4 tumor and the two tumors not represented in **Figure 11**. Noteworthy, the high-molecular weight band was absent in the lanes corresponding to the proteins extracted from the two ganglia, denoting a metastasis-associated profile.

As highlighted by **Figure 11**, samples presenting higher expression of MUC presented low abundance of 25, 15, and 10 kDa proteins (such as the first T1, the second T3 and the T4 tumors visible in the figure). Conversely, samples expressing low amounts MUC presented a high abundance of low-molecular weight proteins (namely the first T2 and T3 and the two ganglia of the figure). It is likely that low-molecular weight bands may correspond to products of proteolysis of MUCs – nevertheless, this hypothesis should be further explored.



High MW glycoproteins (>250 kDa)	+++	+	+	+	-	++	+++	-	-
Low MW glycoproteins (15-25 kDa)	+	+++	+++	+++	+++	+	-	+++	+

(-) not expressed; (+) expressed; the number of "+" symbols relates with the intensity of expression

Figure 11 | STn expression pattern in protein lysates from bladder tumors (T1-T4, n=9) and ganglia (n=2). An intense band at approximately 250 kDa is present in 7 out of the 9 tumors, suggesting its association with mucins, as previously reported by other authors [18, 66, 75]. These bands were not observed in the ganglia. 6 out of the 9 tumors also presented low-molecular weight bands spanning 25-10 kDa. The optical density of the bands was estimated and normalized in relation to beta-actin, previously observed to be a stable housekeeping gene in bladder cancer cells [11]. The bands were then classified in relation to their intensity for comparison purposes. The figure shows that samples presenting higher levels of high-molecular weight proteins also present lower levels of 25, 15, and 10 kDa proteins and vice-versa. These observations suggest that low-molecular weight proteins may be proteolysis products. Noteworthy, lymph node metastasis samples only present low-molecular weight proteins, suggesting a molecular signature associated with metastasis.

The ratio between low-molecular weight proteins and MUC expression in primary tumors was then estimated and the samples were classified as low or high according to their distribution around the median value. As highlighted by **Figure 12**, the majority of

the samples classified as high (n=5) presented metastasis whereas in the group classified as low (n=4) none of the samples presented metastasis, suggesting that the predominance of low-molecular weight species in relation to mucins may dictate the potential for disease dissemination.

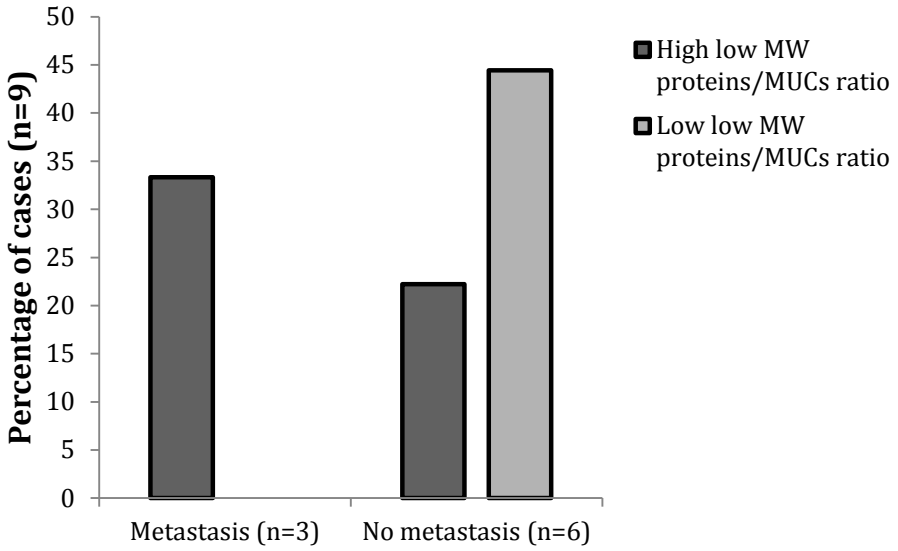


Figure 12 | Ratio between low-molecular weight proteins and MUC expression in primary tumors. As highlighted, the majority of the cases with a high ratio (corresponding to a high expression of low-molecular weight proteins and a low expression of MUCs) developed metastasis. On the other hand, all of the cases presenting a low ratio (high expression of MUCs and low expression of low-molecular weight proteins) did not developed metastasis. The predominance of low-molecular weight species in relation to mucins seems to dictate the ability of the tumor to metastize.

Discussion

The STn antigen is frequently over-expressed in human carcinomas as a reflection of significant alterations in the *O*-glycosylation pathways of cell-surface proteins and the over-expression of heavily *O*-glycosylated proteins, namely mucins. This antigen has been recognized to modulate immune responses and impair protein-protein interactions, promoting higher cell motility and metastic capability [18, 55]. In line with these observations, our group recently reported that the STn antigen was over-expressed in approximately 70% high-grade NMIBC and MIBC, denoting its association with advanced stage bladder tumors, known for an aggressive phenotype and decreased overall survival

[11]. Nevertheless, this study was conducted in rather small and heterogeneous patient cohort (n=69) comprehending low-grade and high-grade non-muscle invasive papillary tumors, CIS and MIBC. The present work now devoted to validate the preliminary findings by Ferreira *et al.* (2013) in a broader series [11], and also to determine the association of STn expression with metastasis.

This work, comprehending a series of 96 NMIBC and MIBC primary bladder tumors, now reinforces the association of STn expression with invasion [11]. Although STn expression has been considerably studied in other carcinomas, namely gastric [63] and colorectal [96], where it is associated with poor prognosis, the assessment of this antigen in a large series of bladder tumors is being addressed for the first time. Culminating these observations, STn-positive cells were observed in invasive areas of the tumor, further suggesting a role of this antigen in invasion. Similar conclusions in an *in vitro* model of gastric cancer were described by Pinho *et al.* (2007) [18]. The authors assessed the role of STn in invasion in human gastric carcinoma cells transfected with ST6GalNAc-I (expressing mostly STn and designated MST6-I), ST6-GalNAc-II (expressing mostly S6T and designated MST6-II) and mock-transfected and reported a 2.5-fold increase in the invasion ability of MST6-I cells when compared to the wild type. Moreover, it was observed an anti-invasion effect by incubation of the cells with anti-STn monoclonal antibody TKH2, confirming that the invasion capability is mediated by the STn antigen. Altogether, our results reinforce the apparent association between STn expression and the invasion ability of bladder tumors, confirming the potential of this antigen as a prognostic biomarker in the bladder cancer context.

The primary bladder tumors were further characterized in relation to proliferation, based on Ki-67 expression, by immunohistochemistry. Ferreira *et al.* (2013) [11], using the same procedure, reported a percentage of 42% Ki-67 positive tumors, in which 38% were high-grade tumors, confirming na high proliferation rate of high-grade tumors. Moreover, since the expression pattern of STn correlated with high-grade tumors, Ferreira *et al.* (2013) [11] highlighted an association between proliferative phenotypes and STn expression. In agreement with these results, we have also observed that all of the STn-positive bladder tumors from our series were Ki-67 positive. Furthermore, in all STn-positive tumors, the antigen expression was mainly seen in areas that did not express Ki-67, although some overlapping areas were present. This indicates that STn is mostly expressed in non-proliferative areas of the tumor, also confirming the observations made by Ferreira *et al.* (2013) [11]. Reflecting the interdependence between STn and Ki-67

expression, the majority of non-proliferative tumors (Ki-67 negative) were also STn-negative. Overall, it can be highlighted that STn is associated with tumor proliferation, characteristic of high-grade tumors.

The STn antigen was also observed in 88% of metastized ganglia and all distant metastasis were positive for this antigen; furthermore, 93% of the correspondent primary bladder tumors were also STn-positive. Altogether, this data suggests that the expression of STn may reflect on the mobility of cancer cells and the capability to metastize. The association of STn expression with metastasis has been demonstrated *in vivo* and also *in vitro* in gastric [75] and breast [97] cancer cells. Namely, Ozaki *et al.* (2012) [75] have assessed the metastatic ability of a human gastric cancer cell line expressing STn in a mouse model. The obtained results showed a prognostic improvement of mice by repeated injections of anti-STn monoclonal antibody B72.3. It was observed an anti-tumor and anti-metastatic effect of B72.3, suggesting the possible involvement of STn in the enhancement of metastatic ability of gastric cancer cells, which is in agreement to our results. In sum, the generated data highlight the association of STn with lymph node and distant metastasis, corroborating the aggressive nature and clinical relevance of this antigen in the bladder cancer context.

Despite the association of STn with a poor prognosis in a variety of cancers [18, 40, 96], scarce information is available about the glycoproteins that carry this post-translational modification [13]. Thus, the characterization of protein expression patterns for STn was established as a secondary goal of this study. In an explorative approach, proteins from 9 primary bladder tumors and 2 metastasized ganglia were evaluated by Western blot for STn expression. An intense staining pattern at 250 kDa is constant among almost all of the primary tumors. This staining is concordant to the presence of mucins, namely MUC1, a high-molecular weight (MW) transmembrane glycoprotein, reported to be heavily *O*-glycosylated and one of the main carriers of STn [18, 66, 75]. Analogous results were reported by Pinho *et al.* (2007) [18], which have identified high MW proteins as the major carriers of STn by Western blot in a human gastric cancer cell line over-expressing this antigen. Furthermore, the authors confirmed through immunoprecipitation that one of the major glycoproteins expressing STn was MUC1. Ozaki *et al.* (2012) [75], using a similar approach, have also identified MUC1 as one of the main carriers of STn in a human gastric cancer cell line expressing STn in a mouse model. These results lead us to hypothesize that the intense staining band at 250 kDa present in our blot refers, possibly among other high MW proteins, to MUC1 mucin. MUC1 over-expressing

the STn antigen in cancer cells is known to suppress cellular aggregation, due to the presence of highly *O*-glycosylated and sialylated structures, which mask the majority of cell-surface molecules and disturb its interaction with the macromolecules on adjacent cell membranes [98]. Moreover, sialylated MUC1 has already shown to inhibit the interaction between cytotoxic lymphocytes and target cells, leading to the apoptosis of lympochytes. This process induces detach of cells from primary lesions, contributing to invasion and metastasis [20, 49]. Therefore, according to the literature, it is suggested that high levels of expression of sialylated MUC1 are associated with an aggressive phenotype, reinforcing our results.

The majority of the cases also presented low MW proteins, namely at 25, 15 and 10 kDa that where decreased in samples expressing high levels of STn in mucins. Conversely, in samples presenting higher expression of these proteins, the levels of mucins were decreased. Altogether, this data also suggests that low MW species may result from the proteolysis of mucins. Noteworthy in the ganglia only low MW proteins could be observed, denoting a molecular profile associated with metastasis. Reinforcing a possible association between the over-expression of low MW proteins with metastasis, the majority of the primary tumors presenting elevated ratios of low MW proteins/MUC presented metastasis, irrespectively of their stage. On the other hand, all cases with low ratio did not present metastasis, including tumors staged T3 and T4. In line with these observations, Lau *et al.* (2012) [50] has recently identified a pathway of EGF (epidermal growth factor)-dependent metastasis that requires a Src (tyrosine-protein kinase)-mediated MUC1 proteolysis, without impact on primary tumor growth. The authors report that EGF stimulation induces MUC1 cleavage, leading the MUC1 cytoplasmic domain transmembrane subunit (MUC1.CD) to translocate to the nucleus, where it promotes the expression of metastatic genes. These results show that EGFR and Src activity, and consequently proteolytic cleavage of MUC1, contribute to tumor metastasis, which is concordant to our results. These observations need to be validated in a large number of patients and efforts should be conducted to identify these glycoproteins and disclose their contribution to malignancy.

In conclusion, our results reinforced previous observations made by Ferreira *et al.* (2013) [11] in respect to the association of STn with invasion and proliferation in bladder cancer. An association of STn with lymph node and distant metastasis was also observed, corroborating the aggressive nature of this antigen and its potential as a prognostic biomarker in the bladder cancer context. We have also observed that high MW proteins

are the major carriers of STn in bladder cancer, in agreement with results from previous studies in gastric [18, 75] and breast [97] cancers. More important, we suggest that a proteolytic cleavage of high MW proteins in bladder tumors originates a pattern of low MW proteins that seems to be associated with a metastatic phenotype.

Chapter V | Patient-derived sialyl-Tn positive invasive bladder cancer xenografts in nude mice: an exploratory model study

Patient-derived sialyl-Tn positive invasive bladder cancer xenografts in nude mice: an exploratory model study

(This chapter corresponds to a manuscript submitted to *Anticancer Research*)

Abstract

Introduction: Over 70% of muscle invasive bladder cancers (MIBC) express the cell-surface antigen sialyl-Tn (STn) that promotes motility and invasive potential of tumor cells. Effective drug testing models to optimize therapy against these tumors are warranted. **Objective:** Validate a patient-derived bladder cancer xenograft model in nude mice to test drugs against STn-positive bladder tumors. **Methods:** Fragments of STn-positive MIBC were subcutaneously engrafted into nude mice and expanded until the third passage. Histology and immunoexpression of tumor markers (p53, p63, Ki-67,CK20,sTn) were analyzed to evaluate tumor phenotype maintenance. **Results:** Tumor take rate was low in the first passage (1/9) but increased and became consistente in the third passage (13/13), therefore suitable for drug testing. Histology and immunoexpression patterns were similar between primary tumor and xenografts. However, p53 and Ki-67 levels increased along passages suggesting a selection of more proliferative clones. STn expression, even though decreased, was preserved in xenografts. **Conclusions:** We describe the first patient-derived STn-positive xenograft model to be used for drug testing and identification of prognostic biomarkers.

Keywords: human xenografts, urothelial cancer, drug testing, animal models, sialyl-Tn, glycosylation.

Abbreviations:

H&E: hematoxylin and eosin; MIBC: muscle invasive bladder cancer; STn: sialyl-Tn; FFPE, Formalin-Fixed, Paraffin-Embedded.

Introduction

Bladder cancer is the fourth most common genitourinary cancer among men and the seventh in women, with an estimated 386,365 new cases and 150,165 deaths each year [99].

Although only one-third of the newly diagnosed bladder carcinomas are advanced at presentation (clinical stage cT2-T4a), 15–30% of high-grade superficial tumors progress to muscle-invasive cancers (MIBC), usually within 5 years [100]. The standard treatment for patients with MIBC is radical cystectomy with removal of regional lymph nodes [101]. However, up to 50% of these patients will relapse with progression to metastatic disease associated with poor survival. [102]. In order to improve this poor outcome, neoadjuvant chemotherapy with therapeutic regimens containing cisplatin, such as MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) or GC (gemcitabine and cisplatin) are recommended [101, 103]. Previous studies showed that neoadjuvant platinum-based chemotherapeutics were associated with an absolute risk reduction of 8% in a 5-years follow-up [104]. However, significant variations in the natural history and responses to treatment are seen between MIBC tumors with identical histological features, reflecting the heterogeneity of the constituent tumor cells [105]. At the moment, there are no biomarkers to predict MIBC response to chemotherapy or assist the design of optimal treatment schemes, which would translate in better outcomes, reduced toxicity and improved overall survival.

Reflecting the molecular heterogeneity of invasive tumors, we recently reported that approximately 70% of MIBC express the sialyl-Tn (STn) carbohydrate antigen, resulting from a premature stop in proteins glycosylation. The STn antigen was observed in highly proliferative tumors and found to promote cell motility and invasive capability [11]. The increased expression of ST6GalNac-I leads to STn biosynthesis [106] in several epithelial cancers (eg. gastric, pancreatic, colorectal, ovarian and breast cancers) and is usually associated with poor prognosis [55, 94] Additionally, the STn antigen contributes to avoid metastatic cell elimination in the blood stream by preventing immune recognition [107], modulates the malignant phenotype [18] and enhances the metastatic ability of cancer cells [75]. Therefore, efficient therapies against STn-positive bladder tumors are warranted; furthermore, the response of these particular tumors phenotypes/clones to available chemotherapy agents remains unknown.

The development of non-human models expressing the STn antigen has been a particularly challenging enterprise. Despite the pan-carcinoma nature of this antigen [55] several well established cancer cell lines of different organs either do not express or lose the capability to present this type of glycosylation [11, 97], denoting a dependence of the tumor microenvironment [11]. In an attempt to overcome this limitation bioengineered cell lines to express the STn antigen have been successfully xenografted into mice and were responsible by enhancing the metastatic capability of cancer cells [18, 97]. Recently, the colon cancer cell line LSC that naturally expresses the STn antigen was also xenografted into nude mice and shown to be inhibited by anti-STn monoclonal antibody 3P9 [108]. However, to our knowledge, the direct xenotransplantation of a STn-positive bladder tumor into nude mice had not yet been attempted.

Xenograft models have been used as standard model predicting efficacy and toxicity of cancer chemotherapeutic agents before entering the clinic due to its ease, low cost, and faster establishment, when compared to the genetic engineered models [109]. In opposition to the xenografts established from cultured cancer cells, where primary cells adapt and suffer a process of natural selection through several passages in culture, direct xenotransplantation of human tumors' fragments preserves original cell heterogeneity, tumor phenotype and malignant potential of human tumors [110, 111]. Patient derived xenografts mimic the heterogeneity of human cancers and have demonstrated superior correlation of chemosensitivity and specificity data for individualized therapy [109] with prediction rates of 90% and 97% for chemosensitivity and chemoresistance, respectively [112]. Previous studies have used cancer xenograft models as a platform for molecular and histopathology studies and therapeutic development with good results in term of success rates, preservation of the original characteristics of the primary tumor and predictive value of the model [113–116]. Preserving the primary tumor characteristics is essential to ensure the original glycosylation patterns, since they are dependent, among other factors, on the way the tumor microenvironment regulates the expression of multiple genes within the glycosylation pathways [117–119]. Still, no evidences have been presented regarding to the preservation of STn expression patterns of the original tumor in direct xenografts.

As such, the goal of this study was to establish a direct human bladder cancer xenograft model in nude mice conserving STn expression of the primary tumor. Such model is regarded of primary importance to identify drugs and treatment regimens that would better serve patients with STn-positive MIBC. It may also be used to test novel therapies and as a platform to identify markers of tumor response and resistance to drugs.

Material and methods

Formalin-fixed paraffin embedded tissues of a MIBC and its first, second and third generation xenografts in nude mice were developed by the Experimental Pathology and Therapeutics Group of IPO-Porto and made available for this work. The clinicopathological characterization of the primary tumor, the animals used for this work, the methodology for the establishment of the xenografts, the histological and immunohistochemistry analysis were as follows:

Primary tumor

A fresh tumor specimen was collected at the time of therapeutic radical cystectomy performed to a 69-year-old man with muscle-invasive urothelial carcinoma (MIBC) at IPO Porto that was neither submitted to preoperative radiotherapy nor neoadjuvant chemotherapy. After surgical excision, tumor tissue was immediately transported to the laboratory in RPMI medium with 1% penicillin/streptomycin. Part of tumor was cut into pieces of 1-2 mm^3 and engrafted in mice while other was fixed in formalin and processed for histological and immunohistochemical analysis.

This study was approved by the ethical committee of the IPO-Porto and informed consent was obtained from the patient.

Animals

The experiments were carried out in accordance with the National and European Convention for the Protection of Animals used for experimental and other scientific purposes and related European Directive (2010/63/EU). Nine male nude mice (strain: N:NIH(s) II-nu/nu), aged 6-7 weeks, obtained from the Animal Experimental Unit at IPATIMUP, Porto, Portugal, were transplanted with human primary tumor. After tumor establishment, 3 and 12 nude mice were used for the second and third passage respectively as illustrated in **Figure 13**. The animals were maintained under sterile conditions throughout the experiment (temperature 24 ± 2 °C, relative humidity 55 ± 5% and a 12 h photoperiod) in polycarbonate cages. They were fed sterilized autoclave rodent feed and water *ad libitum.*

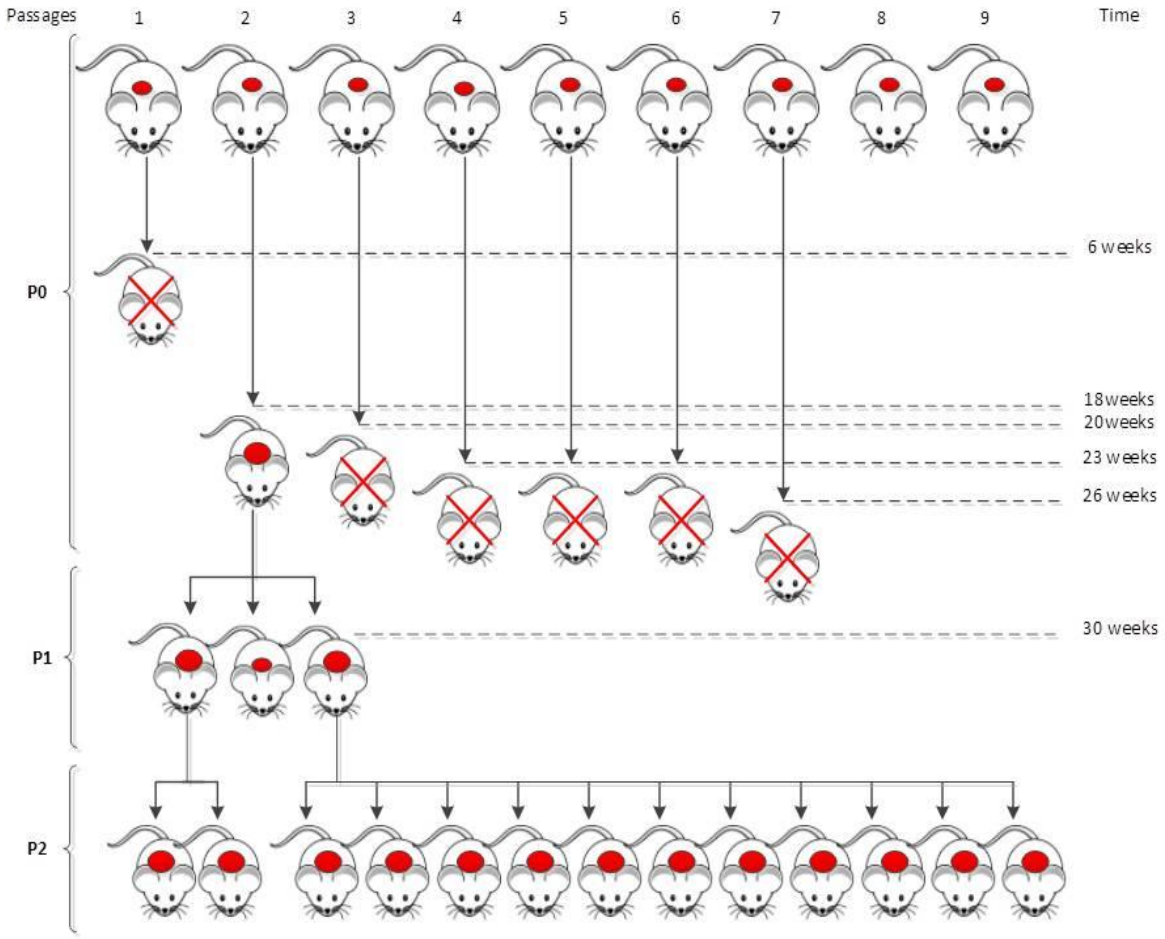


Figure 13 | MIBC tissue was used to establish the xenograft model. After engraftment phase (P0), xenografts were expanded in a group of nude mice as described in Materials and Methods. P1 and P2 represent xenografts of the second and third generations, respectively. The red crosses represents sacrificed mice without tumor growth, the small red balls represents the tumors implanted, whereas the bigger ones represents tumor growth.

Establishment of xenografts

A sample of the patient tumor were cut into 1-2 mm^3 fragments and individual pieces were implanted subcutaneously through small horizontal incisions in the scapular regions of anesthetized nude mice. The tumors were excised when they reached a size of approximately 1.5 cm^3, cut into 1-2 mm^3 fragments and transplanted to another group of mice (P1 and P2) using the same method. Animals were anesthetized using isoflurane

according to the manufacturer's instructions at the time of transplantation and tumor removal.

After transplantation, all mice were observed for development of a palpable mass and tumor growth was assessed using a caliper to determine height(h), width(w) and depth(d) twice a week. Tumor volume was estimated using the formula: $Tumor\ volume = \frac{\pi}{6} h * w * d$ as described elsewhere [120].

Tumor doubling time (DT) was used for quantification of tumor growth rate. DT was calculated as the time period (t) when the tumor volume was twice (v2) the initial volume (v1) during the exponential phase of tumor growth, using the formula: $DT = (t2 - t1)ln2/\ln(\frac{V2}{V1})$ defined by Schwartz [121].

Histological analysis

Tissue from the tumors and mice organs were fixed in 10% phosphate buffered formalin, embedded in paraffin, serially sectioned at 3 μm and stained with hematoxylin-eosin (H&E) for histological examination. Tumors were analyzed in terms of histologic type, degree of differentiation, nuclear atypia and extension of invasion whereas mice organs were accessed for tumor metastasis.

Immunohistochemical analysis

Primary tumor and the tumors grown in the three passages were tested for molecular markers expression through immunohistochemical analysis. Formalin-fixed, paraffin-embedded (FFPE) tumor sections (3 μm) were tested with primary antibodies against p53 (clone DO -7;Dako; 1: 200), p63 (clone 4A4; Dako ; 1:300), Ki-67 (clone Mlb-1; Dako; 1:150), CK20 (clone Ks20.8; Novocastra; 1:150) and STn (anti-STn TKH2 monoclonal antibody [122]; 1:5 from culture supernatant) using polymer-HRP detection method (Power vision). The sections were initially dewaxed in xylene and rehydrated in graded alcohols. Heat-induced epitope retrieval using citrate buffer was carried out according antibody manufacturer's instructions. Endogenous peroxidase activity was inhibited by immersing sections in 0.6% H_2O_2 in distilled water for 20 minutes. Sections were rinsed in PBS-Tween prior to incubation with bovine serum albumin solution (20

minutes) to inhibit non-specific binding. PBS was subsequently used to wash sections between stages. Sections were then incubated with the primary antibodies against p53, p63 Ki-67, CK20 and STn. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer (Power Vision poly-HRP-anti Ms/Rb/R IgG) for 30 minutes and DAB substrate for 7 minutes. Then, the slides were counterstained with hematoxylin and mounted. Positive and negative controls were run simultaneously with tumor specimens. The expression of STn was further validated by observing the loss of reactivity with anti-STn monoclonal antibody TKH2 after treatment of the tumor with a neuraminidase from *Clostridium perfringens* (Sigma-Aldrich), as previously described by Marcos *et al.* [94]. This treatment was responsible by removing the sialic acid from STn which impaired antibody recognition.

The staining patterns were assessed by two independent observers (one of them a pathologist) using standard light microscope. Positive staining was considered when more than 10% of tumor cells showed reactivity. Stain intensity and percentage of tumor cells stained were recorded to each tumor marker and classified into categories A (< 25%), B (25-50%), C (50-75%), D (>75%) according to the number of positive tumor cells stained. The Wilcoxon sign rank test was applied to disclose differences between the levels of expression of tumor markers in the primary tumor and xenografts using Stata 12.1 for Windows (Stata Corp LP, Texas, USA).

Protein extraction and Western blot

Proteins were extracted from FFPE tissues using the Qproteome FFPE tissue kit (Qiagen). The amount of protein in each extract was estimated with DC protein assay kit (BioRad). Thirty micrograms of protein were separated by 4–16% gradient SDS–PAGE under reducing conditions and transferred onto 0.45 μm nitrocellulose membranes (GE Healthcare). Membranes were blocked with 1% carbohydrate depleted carbo-free solution (Vector Laboratories) for 1 hour at room temperature, incubated overnight at 4ºC with anti-STn TKH2 monoclonal antibody in culture supernatant, washed with TBS-T for 30 minutes, and finally incubated for 45 minutes with goat anti-mouse IgG1 heavy chain horseradish peroxidase conjugate (Abcam; 1:35,000 in TBS). After washing, the bound antibodies were revealed by chemiluminescence using the ECL prime Kit (BioRad). Samples previously de-sialylated with as previously described by Marcos *et al.* [94] were used as controls.

Results

A direct human bladder cancer xenograft model was established (P0) and expanded (P1) in a group of nude mice until the third passage (P2), as schematized in **Figure 13**.

The primary tumor used to establish the xenografts was obtained after radical cystectomy performed to a 69-year-old man diagnosed with invasive urothelial bladder cancer (pT3aN0M0). Histologic analysis presented a high grade urothelial carcinoma invading the muscularis propria and perivesical fat (pT3a). Regional lymph nodes and surgical margins were tumor free. Preliminary analysis by immunohistochemistry showed an intense and diffuse STn-expression pattern throughout the tumor (>70% positive) including in cells invading the muscle and fat layers. Few cells in the tumor-adjacent mucosa were also positive whereas stromal cells were negative. Staining was observed in the cytoplasm, mainly in the *trans* Golgi region and was particularly intense in the cell membrane, thus in accordance with our previous observations [11].

In the first passage (P0), one out of nine mice presented tumor growth, which corresponded to a success take rate of 11%. The percentage of successful engraftment increased in sequential passages (2/3 in P1 and 12/12 in P2). The lag period was 4 months for the first passage, and became shorter in subsequent passages, 5-9 weeks in the second and around 9 weeks in the third (**Figure 14**). Of note, despite some variance in the lag period between xenografts in the third passage, the tumor growth curves became more similar and constant among them, suggesting consistency in tumor growth. Tumor doubling time was approximately 6 days in the first generation and became longer in subsequent passages, around 10 and 14 days in the second and third passage respectively. The mice bearing tumors in the three passages had no macroscopic evidence of invasion of adjacent tissues at the time of tumor excision neither metastasis were found at the time of sacrifice.

The establishment of tumors with high successful take rate and homogeneous growth, suitable for drug testing studies, was achieved at the third generation (P2) 8 months after the xenotransplantation of the primary tumor.

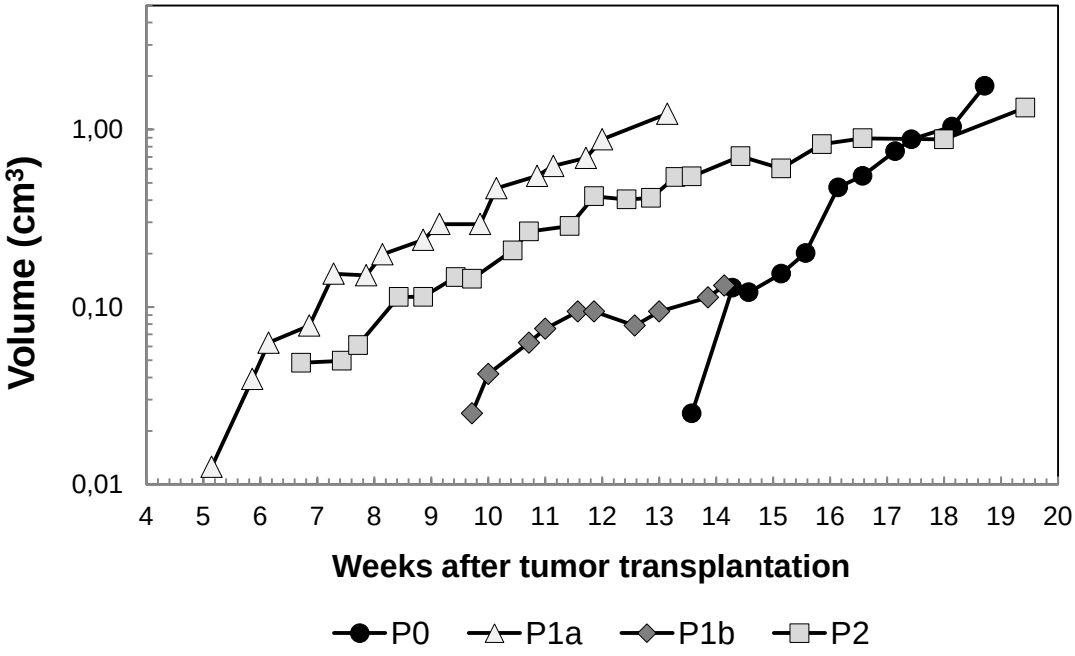


Figure 14 | Tumor growth curve for the first (P0), second passage P1 (a and b) and third generation (P2) xenografts. In the first passage the xenograft presented a lag period of 13.5 weeks before progressive tumor growth was observable. The time between transplantation and palpable tumor growth became shorter on the subsequent passages, 5 (P1a) and 9 (P1b) weeks in the second passage and 6 weeks in the third. Xenografts growth rate was regular in the three passages. Different lag periods observed for P1 (P1a and P1b) denote instability in the adaptation of the tumors to the host in the second generation. Both the tumor lag periods and growth rates became stable in the third generation. Tumor volume was plotted on a logarithmic scale.

Histological and immunohistochemical analysis of the primary tumor and xenografts

The morphological characteristics of xenografts were analyzed and compared with the primary tumor. The histology of the original tumor revealed an invasive urothelial bladder carcinoma with high nuclear-to-cytoplasmic ratio, nuclear atypia and presence of mitotic figures. The resulting xenografts presented identical histologic features to those observed in the primary tumor, particularly in terms of cellular type and grade of atypia. The percentage of necrosis was around 10% in both primary tumor and xenografts and some xenografts presented scamous differentiation that ranged between 10 and 25%.

In addition to the neoplasic cells, the first passage xenograft also presented a cyst covered by a layer of epithelial like cells, which also enclosed the tumor. This vesicle-like sac was composed by an epithelial lining layer of variable number of cells and dense connective tissue and represents the heterogeneity of the tumor cells implanted. This structure was no longer present in subsequent passages, denoting some degree of clonal selection for malignant cells.

The primary tumor and xenografts were further evaluated by immunohistochemistry in relation to proliferation (Ki-67), aggressiveness (p53) and differentiation (p63 and CK20) markers (**Figure 15**) and the STn antigen expression (**Figure 16**), whose over-expression is common in aggressive bladder cancer [123].

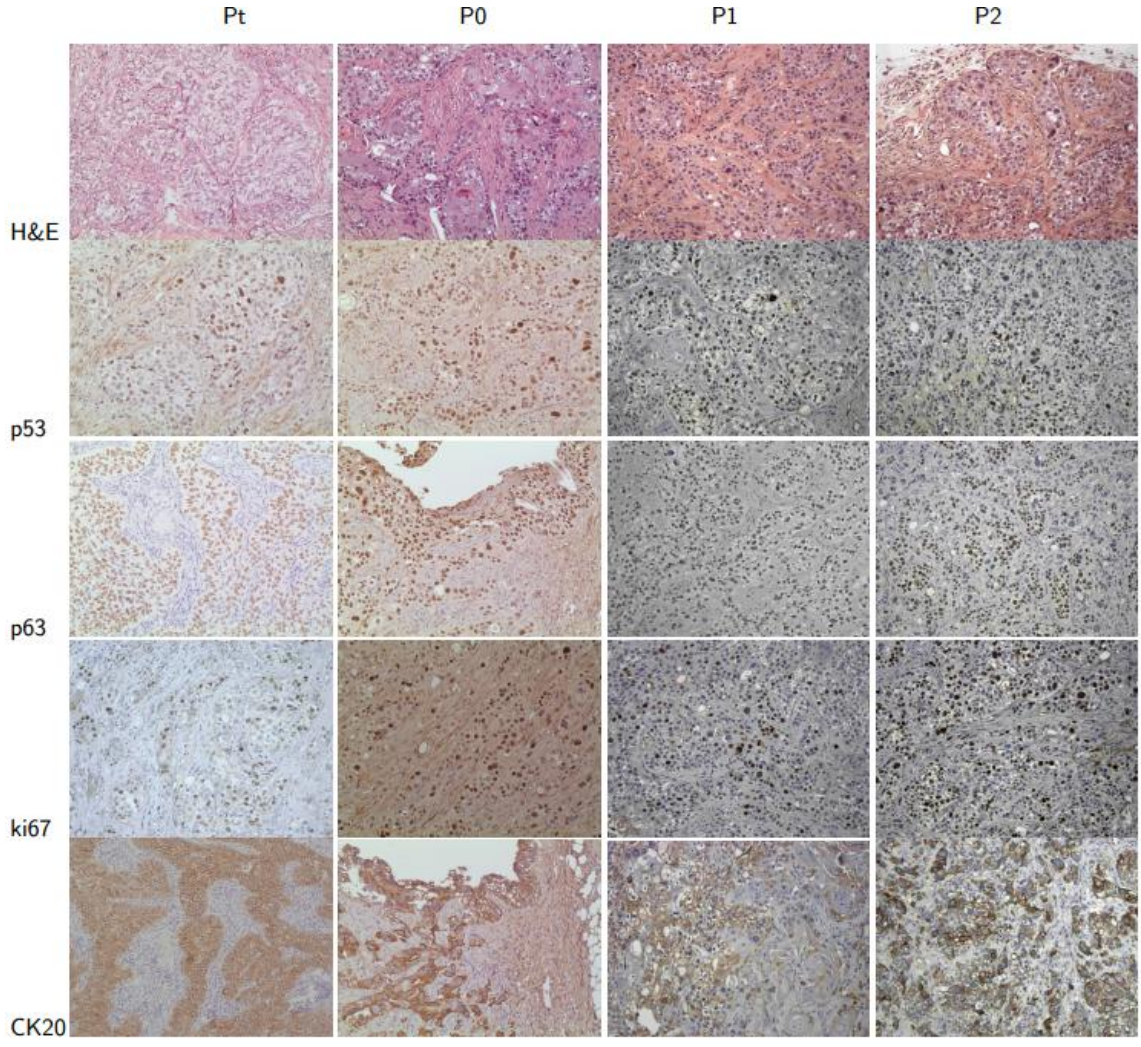


Figure 15 | Histology and tumor molecular markers (p53, p63, Ki-67, CK20) immunoexpression of primary tumor (Pt) and first (P0), second (P1), third (P2) generation xenografts (original magnification x200). No major differences are seen in the tumor structure and cancer cells markers expression between the original tumor and the xenografts. However in the

case of p53 and Ki-67 expression, an increase was observed in xenografts when compared with the original tumor, suggesting a more aggressive and proliferative phenotype.

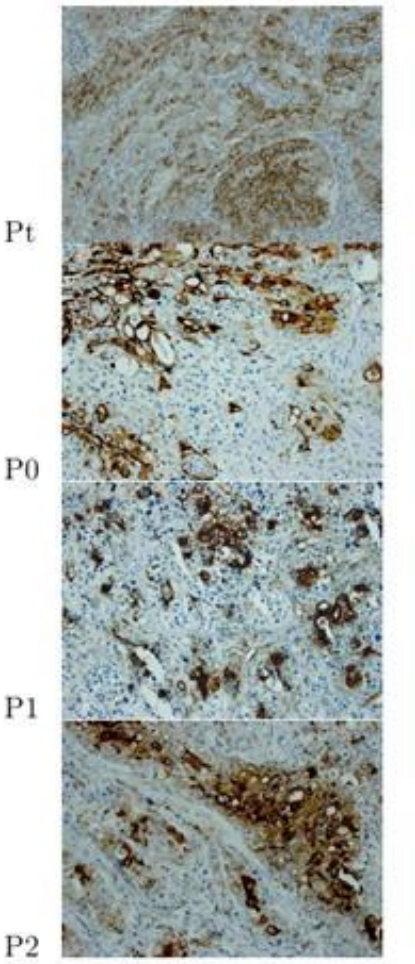


Figure 16 | A) STn immunoexpression in primary tumor and xenografts in first (P0), second (P1) and third generations (P2) (original magnification x200). STn expression was maintained, even though decreased, in the xenografts.

Both the primary and the xenografted tumors (P0, P1 and P2) were positive for these markers, reinforcing the homology suggested by histological analysis (**Figures 13** and **14**). Likewise, the primary tumors and the xenografts shared a strong and diffuse expression of p63 and CK20 (>75% of the tumor area), representing similar degrees of differentiation (**Table 2** and **Figure 17**). However, significant variations were observed in the levels of p53, Ki-67 and STn between the primary tumor and xenografts and also between sequential passages (**Figure 17**). In general, the percentage of p53 positive cells was higher in xenografts when compared to the primary tumor (25-75% and 10-25% respectively). This tendency was particularly pronounced in the third generation

xenografts. The levels of Ki-67 immunoexpression were also significantly elevated in P0 in comparison to the primary tumor (50-75% and 25-50% respectively). Some variations in Ki-67 expression were also observed between xenografts in different passages (**Figure 17**). The P0 presented the highest proliferative index, in agreement with the lower tumor doubling time presented by the first generation xenografts (**Figure 13**). The expression of Ki-67 decreased in P1 to levels similar to the initial tumor but increased again in P2. Altogether, these data suggests that the xenograft establishment process may be accompanied by the selection of a more aggressive and proliferative phenotype characterized by a significant over-expression of Ki-67 and p53 (**Table 2**).

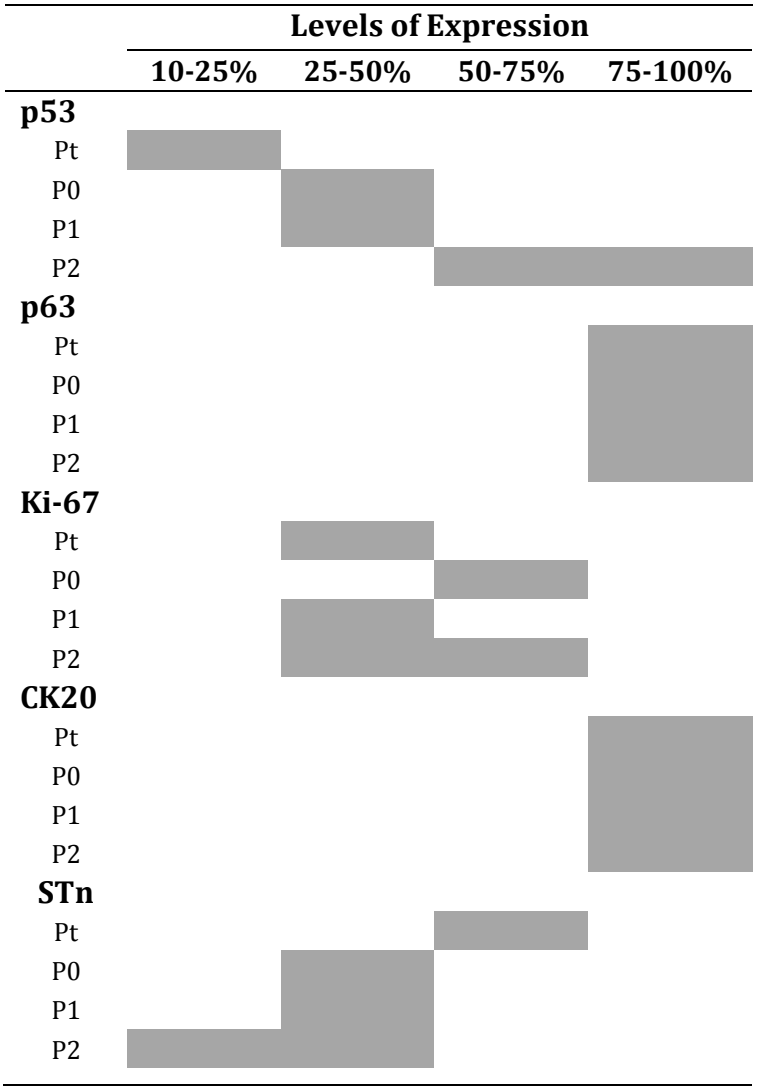


Figure 17 | Immunoexpression of tumor markers p53, p63, Ki-67, CK20 and STn in the primary tumor (Pt) and first (P0), second (P1) and third (P2) generation xenografts. The expression levels of p63 and CK20 presented by the primary tumor were conserved in the

xenografts denoting similar levels of differentiation. The expression of p53 was increased in the xenografts in relation to the primary tumor and increased with sequential passages. The Ki-67 increased in P0 but decreased in P1 denoting a process of adaptation of the tumor cells to the host. The proliferation then increased in P2, suggesting the selection of proliferative clones. Altogether, STn antigen expression was decreased in the xenografts in relation to the primary tumor and decreased with the passages.

Table 2 | Comparison between the immunoexpression of tumor markers p53, p63, Ki-67, CK20 and STn in the primary tumor and the third generation xenografts (P2), that showed high and homogeneous growth rates.

Levels of Expression	Primary Tumor	P2 Xenografts	
p53			
A	1 (100%)	1 (8%)	
B	0	4 (33%)	*p*-value
C	0	6 (50%)	0.0021
D	0	1 (8%)	
p63			
A	0	0	
B	0	0	*p*-value
C	0	1 (8%)	0.3173
D	1 (100%)	11 (92%)	
Ki-67			
A	0	0	
B	1 (100%)	6 (50%)	*p*-value
C	0	6 (50%)	0.0143
D	0	0	
CK20			
A	0	0	
B	0	0	*p*-value
C	0	1 (%)	0.3173
D	1 (100%)	11 (92%)	
STn			
A	0	5 (42%)	
B	0	6 (50%)	*p*-value
C	1 (100%)	1 (8%)	0.0020
D	0	0	

[a] Wilcoxon sign rank

Percentage of expression: A (< 25%), B (25-50%), C (50-75%), D (>75%)

The STn antigen, that was highly expressed in the primary tumor (>75% of the area), was also detected in xenografts (**Figure 16**); however its levels decreased with xenotransplantation and along consecutive passages. Since the STn antigen is a post-translational modification common to several cell-surface glycoproteins, we have also evaluated whether the protein pattern of expression remained conserved in the P2 xenografts by western blot (**Figure 18**). Both blots presented a dominant band at approximately 55 kDa and several high-molecular weight bands above 75 kDa that were no longer observable in control experiments with desialylated protein extracts. Therefore, even though decrease in relation to the primary tumor, the expression pattern of STn remains conserved in the P2 xenografts proteins (**Table 2**).

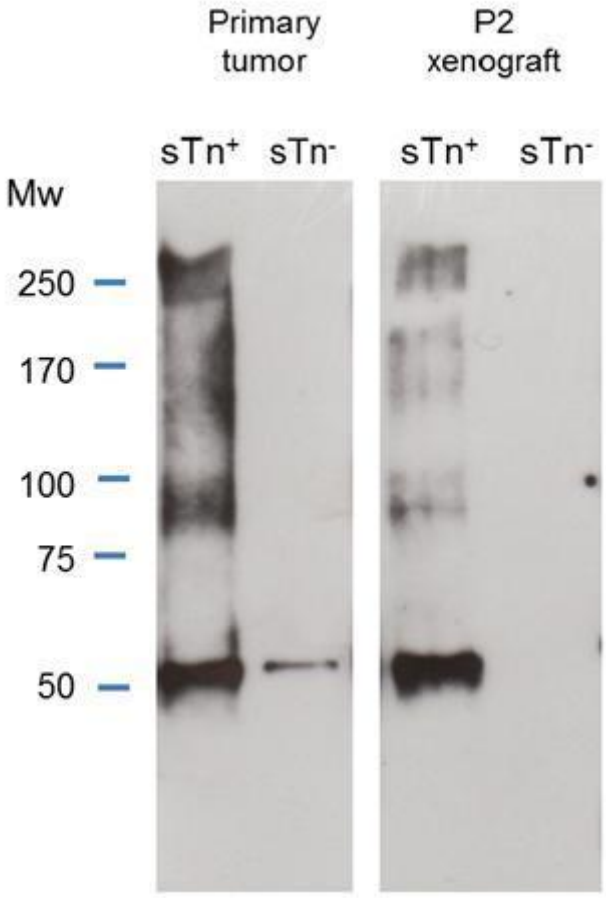


Figure 18 | Western blot for the proteins expressing the STn antigen in primary tumor and P2 xenografts. Similar protein patterns were observed in both cases; differences in staining intensities are thought to results from the lower expression of STn in xenografts. STn+ refers to the native protein extracts while STn- refers to protein extracts after desialylation with a neuraminidase, which impairs recognition by anti-STn monoclonal antibody. The absence of immunoreactivity in the STn- bands confirms the specificity of the recognition pattern.

Discussion

We recently reported that a significant percentage of MIBC expressed the STn carbohydrate antigen, a post-translational modification of cell-surface proteins responsible by enhancing the motility and invasive capability of bladder cancer cells [11]. Therefore, the goal of this study was to establish a direct human bladder cancer xenograft model in nude mice conserving STn expression of the primary tumor. Such model is regarded of primary importance to identify drugs and treatment regimens [124] that would better serve patients with STn-positive MIBC.

Herein, we describe the transplantation of freshly collected STn-positive MIBC fragments directly into nude mice that were then passed to other mice without compromising the histological and molecular nature of the original tumor. Tumor take rate and the consistency in growth rates following implantation, critical aspects of xenograft models, have also been evaluated. In our study, one sample of MIBC was implanted into 9 nude mice with a success take rate of 1/9 (~11%) that increased in subsequent passages. Analogous success rates have been described in previous studies using similar methods for establishing bladder cancer xenografts, independent of primary tumor stage. Namely, Hay *et al.,* that reported a success take rate of 7/48 (15%) after bilateral transplantation of bladder cancer specimens into 5 immunocompetent mice previously subjected to thymectomy and whole body radiation [125]. Kovnat *et al.*, using the same procedure reported a success take rate of 8/33 (24%) and 20/53 (38%) [126, 127]. More recently, Abe *et al.* reported an overall success rate in xenograft establishment of 62.5% (15/24) using severe combined immunodeficient mice and one or two fragments of 4-5 mm^3 per mice [128]. In order to increase the tumor take rate, some aspects may be modulated in future xenografts, specifically the number and volume of the fragments implanted.

The lag period was 4 months for the first generation xenografts and became shorter on the subsequent passages, 5-9 weeks in the second and around 9 weeks in the third. The longer lag period observed in the first passage might be explained by the presence of a low fraction of clonogenic cells in the fragment implanted and the need to adapt and grow in a new environment. The xenografts growth rates were also different between passages, however, they became similar between xenografts of the same passage in the third generation (P2), suggesting growth consistency. This aspect enables comparison of tumor growth between groups and assessment of tumor response to chemotherapeutic drugs.

These results are in agreement with a previous reports using similar methods (human bladder cancer specimen nude mice and subcutaneous implantation) to obtain xenografts from bladder tumors [128] as well as with other cancer xenograft models [111, 116, 124, 129].

Histological analyses have demonstrated high similarity between primary tumor and xenografts in terms of cellular type and grade of atypia, suggesting that the phenotype of the primary tumor is preserved during tumor establishment and expansion in nude mice. This observation was reinforced by the detection of similar levels of differentiation markers p16 and CK20. However, variations in the expression of p53 and Ki-67 were observed between the primary tumor and the xenografts and between different passages. A comparison between primary tumor and third generation xenografts has further highlighted an increased expression of p53 and Ki-67, suggesting a tendency to select of the most aggressive and rapidly growing cells from a heterogeneous primary tumor during engraftment process. In accordance with these observations, several publications have described that certain cell populations of the primary tumor can be amplified by the xenografting process and may represent the natural tumor evolving process towards a more aggressive phenotype with higher potential to adapt and metastasize [130, 131]. Along with the ability to obtain high take rate and stable tumor growth, these events reinforced the value of third generation xenografts and subsequent passages as a good model for cancer drug testing.

We also observed that xenografts reproduced the STn expression pattern observed in the primary tumor, thereby creating the first *in vivo* bladder tumor model expressing this antigen. The development of non-human models expressing the STn antigen has been a particularly challenging enterprise. Despite the pan-carcinoma nature of this antigen [55], in vivo models that mimic clinical setting of tumors expressing this antigen are still missing and the direct xenotransplantation of a STn-positive human bladder tumor cells into nude mice had not yet been attempted. In our model STn antigen expression decreased in xenografts in comparison to the primary tumor, a decrease that became more pronounced in the third generation which also showed a more prominent proliferative phenotype. Such observations reinforce our previous findings that, despite being associated with proliferative phenotypes, cells expressing the STn antigens are commonly found in non-proliferative invasive areas of the tumor. Nevertheless, the third generation xenografts conserved significant STn expression and presented an STn-expressing glycoprotein profile similar to the primary tumor. Studies are ongoing to determine the

nature of these proteins and disclose the environmental factors that promote STn expression in bladder cancer.

Given their capability to recapitulate the histological and molecular nature of the primary tumor, the STn-expressing bladder cancer xenografts show potential as a model to determine the adequate treatment schemes for these tumors, test new drugs and identify prognostic biomarkers. Similar xenograft models for other cancers have shown a "remarkable correlation between drug activity in the model and clinical outcome" and have already been transpose into the clinical practice [116]. Altogether we believe that this approach may also be useful in the context of MIBC. Additional work involving a larger number of patients is ongoing to optimize the model and further explore its potential in drug testing. Given the pancarcinoma nature of STn antigen expression, its association with invasion and metastases, these findings may constitute valuable insights for other tumors.

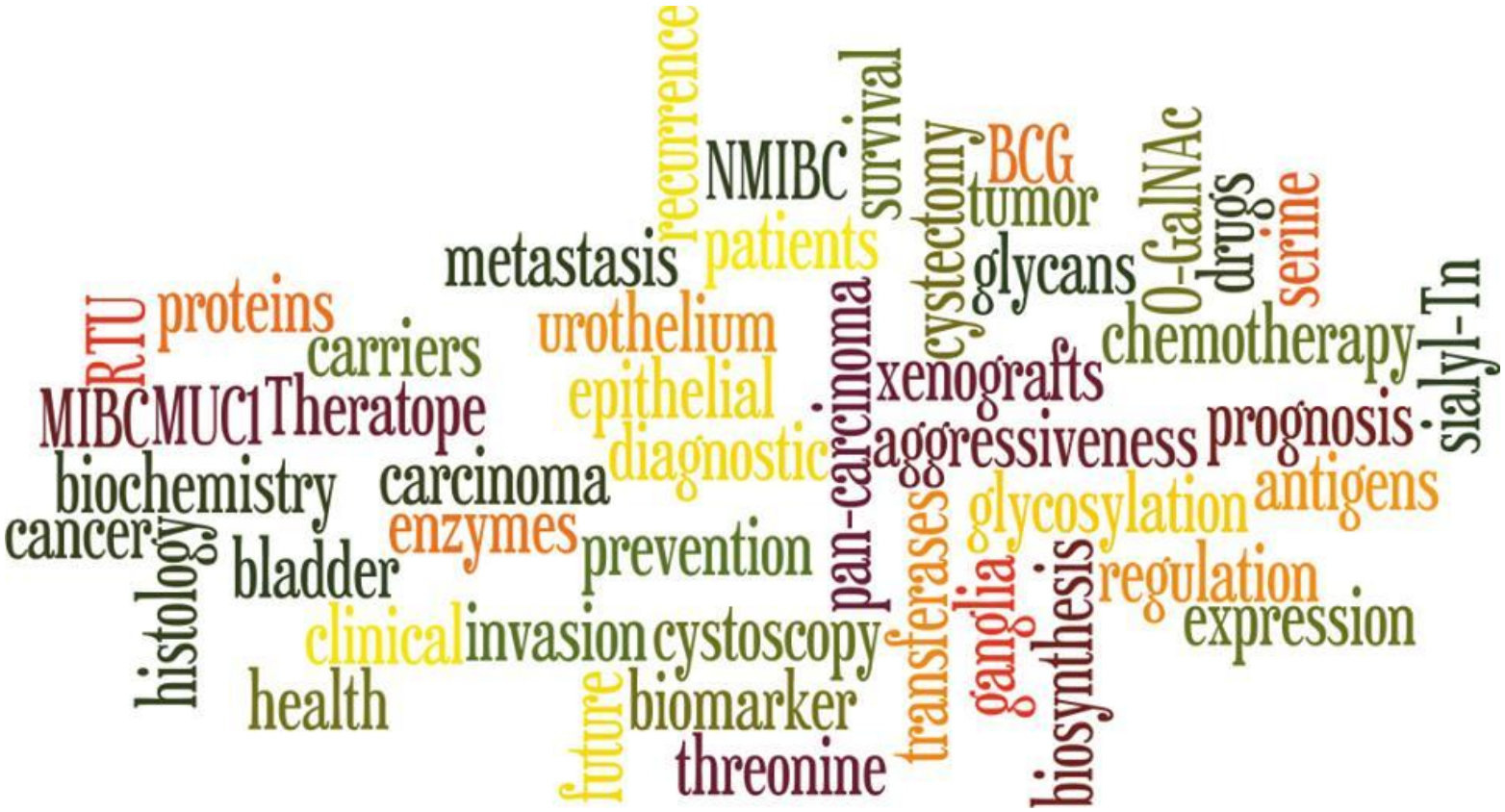
Chapter VI | Conclusions

Conclusions

Aiming to determine the association of STn with invasion, proliferation and metastasis, primary bladder tumors and lymph node and distant metastasis were screened for the STn expression, by immunohistochemistry. This confirmed the association of STn with the referred clinical settings, corroborating the aggressive nature of this antigen and its potential as a prognostic biomarker in bladder cancer. Furthermore, it was also confirmed by Western blot that high-molecular weight proteins are the major carriers of STn in primary tumors. Conversely, metastasis showed a pattern composed of low-molecular weight proteins (<25 kDa), that appear to result from the proteolytic cleavage of high-molecular weight species. Future studies should be conducted to disclose a possible metastasis-associated glycoprotein profile.

The second part of the work aimed to validate a STn-expressing bladder cancer xenograft as a model for drug testing and identification of prognostic biomarkers. It was observed that xenografts retained the histological nature of the primary tumor and similar levels of differentiation markers p63 and CK20. However, p53 and Ki-67 levels increased along passages while STn expression decreased, suggesting a selection of the most proliferative clones. Nevertheless, the glycoproteins STn-expression pattern of the primary tumor was conserved in the xenografts. Taken together, the generated data suggests that xenografts mimic the molecular nature of the primary tumor, including STn expression.

Altogether, important insights have been provided about the clinical relevance of STn in bladder cancer that will help guiding the development of novel STn-based therapies.



Chapter VII | Future work and perspectives

Future work and perspectives

This work has provided important insights about the clinical relevance of STn in bladder cancer. Future studies should be conducted in a larger number of patients including different clinicopathological features, such as precursor lesions, carcinoma *in situ* (CIS) and inflammatory disease, to isolate cancer-associated patterns. Associations with response to therapeutics and the expression of other biomarkers of aggressiveness disease may also help to determine the group of patients better served by an STn-based therapeutics.

Moreover, the identification of the STn expressing proteins will be necessary to disclose the biologic role of this antigen. It would be also imperative to determine the nature of the low-molecular weight proteins and their role in metastasis. The validation of a protein profile associated with metastasis will also require the evaluation of a larger number of patients, including samples of metastasis. Altogether, this information will create the clinical basis for the development of therapeutics for aggressive bladder cancer malignant disease.

The development of novel drugs requires animal models able to mimic the primary tumor. This work suggests that patient derived STn-positive xenografts may reflect the histologic and molecular nature of the primary tumor, however showing signs of clonal selection towards more aggressive proliferative phenotypes. The validation of such a model requires the histologic, proteomic and genomic comparison between the model and the primary tumor in a larger number of patients. Given the pan-carcinoma nature of STn antigen expression and its association with invasion and metastasis, the optimization of the model may constitute valuable insights for other tumors.

Chapter VIII | Bibliography

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