Maria do Céu dos Santos Silva Costa

Carcinoma da Bexiga: Via de Sinalização PI3K/Akt/mTOR e Inibidores do mTOR

Tese de candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Prof. Doutor Lúcio José de Lara Santos

Categoria – Professor Auxiliar Convidado

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Este trabalho foi realizado no Grupo de Patologia e Terapêutica Experimental Centro de Investigação do Instituto Português de Oncologia do Porto, e na Unidade de Investigação FP-ENAS da Universidade Fernando Pessoa.

Em obediência ao disposto no nº 2 do Artigo 31º do Decreto-lei nº 178/2009, o autor declara que participou na conceção e na execução do trabalho experimental, bem como na interpretação dos resultados e na redação dos seguintes trabalhos publicados e em publicação, que fazem parte integrante desta Dissertação:

Costa C and Pereira S, Lima L, Peixoto A, Fernandes E, Neves D, Neves M, Gaiteiro C, Tavares A, Gil da Costa R, Cruz R, Amaro T, Oliveira P, Ferreira A and Santos L. Abnormal protein glycosylation and activated PI3K/AKT/mTOR pathway: role in bladder cancer prognosis and targeted therapeutics. Artigo aceite para publicação na revista PLOS ONE.

Ferreira JA, Videira Pa, Lima L, Pereira S, Silva M, Carrascal M, Severino PF, Fernandes E, Almeida A, Costa C, Vitorino R, Amaro T, Oliveira MJ, Reis CA, Dall'Olio F, Amado F, Santos LL. (2013) Overexpression of tumour-associated carbohydrate antigen Sialyl-Tn in advanced bladder tumours. *Mol Oncol*. Jun;7(3):719-31. doi: 10.1016/j.molonc.2013.03.001.

Padrão AI, Oliveira P, Vitorino R, Colaço B, Pires MJ, Márquez M, Castellanos E, Neuparth MJ, Teixeira C, Costa C, Moreira-Gonçalves D, Cabral S, Duarte JA, Santos LL, Amado F, Ferreira R. (2013) Bladder cancer-induced skeletal muscle wasting: disclosing the role of mitochondria plasticity. Int J Biochem Cell Biol. Jul;45(7):1399-409. doi: 10.1016/j.biocel.2013.04.014.

Bernardo C, Costa C, Sousa N, Amado F, Santos L. (2015) Patient-derived bladder cancer xenografts: a systematic review. Transl Res. Feb 12. pii: S1931-5244 (15) 00040-7. doi: 10.1016/j.trsl.2015.02.001.

Bernardo C, Costa C, Amaro T, Gonçalves M, Lopes P, Freitas R, Gärtner F, Amado F, Ferreira JA, Santos L. (2014) Patient-derived sialyl-Tn-positive invasive bladder cancer xenografts in nude mice: an exploratory model study. Anticancer Res. Feb;34(2):735-44.

Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, Colaço B, Lopes C, Colaço A, Costa C, da Silva VM, Oliveira P, Santos L. (2013) Everolimus combined with cisplatin has a potential role in treatment of urothelial bladder cancer. Biomed Pharmacother. Mar;67(2):116-21. doi: 10.1016/j.biopha.2012.11.007.

Dedico este trabalho aos meus Pais,

Que tanto fizeram por mim…

Agradecimentos

Desde o início desta longa etapa, contei com o apoio, incentivo e confiança de inúmeras pessoas e instituições a quem quero deixar aqui os meus sinceros agradecimentos:

Ao meu orientador, Professor Doutor Lúcio Lara Santos, pelos seus ensinamentos sempres sábios, generosos e pacientes, mas também pela sua exigência, rigor, apoio e amizade que demonstrou no decorrer deste trabalho e ao longo destes anos.

À Dra. Teresina Amaro, pelo auxílio na avaliação de muitos dos resultados de imunohistoquímica.

À Professora Doutora Paula Oliveira pela cedência do seu material, pelo apoio constante e disponibilidade ao longo desta etapa.

À Professora Doutora Paula Soares pelos conhecimentos transmitidos e preciosa ajuda na escolha do caminho a seguir.

Ao Professor Doutor Gil da Costa por toda a sua disponibilidade, incentivo e confiança demostrada.

À Professora Doutora Rita Ferreira pela sua ajuda e profissionalismo que tão importante foi para este trabalho.

Ao Professor Doutor Alexandre Ferreira por toda a sua ajuda sobretudo na reta final do trabalho.

À Universidade Fernando Pessoa que me acolheu para a realização deste doutoramento, e em Particular ao seu Reitor, o Professor Doutor Salvato Trigo, por permitir a realização da maioria das tarefas laboratoriais no centro de investigação FP-ENAS.

À Mestre Sofia Pereira pela troca de ideias contantes.

À Mestre Carina Bernardo por todo o apoio, ajuda e compreensão… Muito obrigado Carina.

Ao Professor Doutor Carlos Palmeira, em primeiro lugar pela sua amizade demonstrada ao longo destes anos e em segundo pela ajuda incansável e disponibilidade que sempre demonstrou.

À Professora Doutora Sandra Clara Soares, por toda a ajuda na escrita deste trabalho, pela sua disponibilidade e grande amizade demonstrada. Pelo apoio constante principalmente nos momentos mais críticos. Nunca mais esquecerei…

À Professora Doutora Rita Castro, pela sua ajuda e disponibilidade permanente, mas principalmente pela sua amizade e encorajamento constante.

À Professora Doutora Cristina Abreu pelos seus conselhos sempre na hora certa.

À Mestre Sílvia Cunha pela força transmitida e por me fazer acreditar que era possível, obrigado pela tua amizade.

Aos meus sogros, pela ajuda incansável e auxílio constante que sempre demonstraram.

Um agradecimento especial ao meu marido, Rui, por tudo.

Às minhas filhas Inês e Carolina, pelo tempo roubado que por direito lhes pertencia.

À minha irmã Margarida e toda a família.

Agradeço ao meu Pai e à minha Mãe por tudo o que fizeram por mim…Como eu gostava que estivessem a presenciar este momento…Amo-vos muito…

E a todos que direta ou indiretamente fizeram parte deste percurso, o meu muito obrigado.

Resumo

O carcinoma urotelial da bexiga (CUB) é um grave problema de saúde, com elevada taxa de morbilidade e mortalidade devido à sua elevada heterogeneidade tanto no comportamento clinico como na histogénese. Os protocolos terapêuticos de rotina não são eficazes para todos os doentes com CUB tanto superficial como invasor. Neste momento novos fármacos estão em desenvolvimento, nomeadamente inibidores do mTOR, com novos alvos terapêuticos.

O alvo da rapamicina nos mamíferos mTOR (mTOR), a jusante da via de sinalização fosfatidilinositol 3-cinase (PI3K)/Akt, possui um papel chave na regulação do metabolismo celular, crescimento, proliferação e sobrevivência; alterações nesta via são comuns em variadas patologias oncológicas, nomeadamente no carcinoma da bexiga.

A glicosilação alterada ou aberrante é comum a vários tipos de carcinomas, nomeadamente durante a progressão tumoral alterando a adesão e mobilidade celular, promovendo a invasão e metastização. A glicosilação aberrante é responsável pela expressão de carbohidratos associados à malignidade e é catalisada por glicosiltransferases e glicosidases específicas. O antigénio sialino – STn, associado ao cancro é um produto aberrante da glicosilisação, expresso em variados tipos de carcinomas, como ovárico, gástrico, colon e carcinoma da bexiga. A existência de uma possível correlação entre a expressão do STn e a via de sinalização mTOR em tumores avançados poderá ajudar na estratificação de doentes bem como no desenvolvimento de novas estratégias terapêuticas.

O objetivo principal deste conjunto de estudos foi avaliar a expressão de moléculas da via de sinalização PI3K/AKT/mTOR e do STn nos tumores humanos e correlacionar a sua expressão com o prognóstico dos doentes.

Observámos que moléculas a montante e a jusante da via PI3K/AKT/mTOR demonstravam igual expressão comparando carcinomas da bexiga não musculo-invasores e musculo-invasores e não se relacionavam com o estado da doença; a expressão de PTEN estava diminuída em tumores avançados relacionando-se com uma baixa sobrevida. Também, encontramos uma associação entre a expressão de STn e a invasão muscular, correlacionada com a sobrevida. Esta tendência

também foi observada em tumores com expressão de pAkt, pmTOR e/ou pS6 nos grupos STn positivos nos tumores musculo-invasores.

Posteriormente foi avaliada a expressão de moléculas da via de sinalização PI3K/AKT/mTOR e do STn em linhas celulares e modelos animais para perceber o efeito dos inibidores mTOR na proliferação e sobrevivência das células tumorais.

Nos carcinomas uroteliais da bexiga induzidos quimicamente em roedores fomos analisar o impacto do sirolimus - um inibidor do mTOR na progressão tumoral. Verificámos que os roedores tratados apresentavam um menor número de lesões invasivas em paralelo com a expressão diminuída de pS6 e STn. Pela primeira vez descrevemos que a administração de sirolimus é eficaz em células STn positivas, o que mostra uma possível ligação entre a via de sinalização PI3K/AKT/mTOR e o STn em tumores invasivos.

Relativamente aos inibidores do mTOR avaliámos a eficácia da combinação everolimus (RAD001) com cisplatina em linhas celulares de carcinoma urotelial da bexiga. Observámos que esta combinação levava à diminuição da proliferação celular num *racio* dependente da dose, em comparação com o fármaco sozinho, nomeadamente nos tumores não invasores da camada muscular.

Fomos também avaliar de que forma a expressão de moléculas da via de sinalização PI3K/AKT/mTOR e do STn se correlacionam com o crescimento tumoral e caquexia.

Em relação à via de sinalização PI3K/AKT/mTOR e a perda de peso muscular nos CUB, verificámos, em roedores, que havia uma perda de 17% no peso-caquexia; os níveis diminuídos de pAKT, mTOR e p70S6K sugerem uma supressão da via PI3K/AKT/mTOR.

Observando a correlação da expressão de STn nos CUB com proliferação tumoral e a sua relevância clinica, vimos que 75% dos tumores de alto grau que expressavam STn apresentavam elevados níveis de proliferação celular e alto risco de recorrência/progressão. A expressão de STn correlacionou-se com a capacidade invasiva e esta não era detetada no urotélio normal.

Relativamente aos estudos usando ratinhos xenógrafos fomos estudar o modelo tumoral de CUB. Olhando para a expressão dos marcadores tumorais p53, p63, KI-67, CK20 e STn

encontramos similaridades entre os tumores primários e os xenógrafos embora a expressão de p53 e KI-67 se encontrasse aumentada; nos xenógrafos a expressão de STn mantinha-se apesar de diminuída fazendo deste um bom modelo a usar para estudos de CUB.

Em suma, os nossos resultados demonstram a ativação da via PI3K/AKT/mTOR em carcinomas não-invasores e invasores da camada muscular; esta é um fator chave na carcinogénese urotelial da bexiga e como tal, os inibidores do mTOR apresentam um grande potencial como novas drogas neste tipo de carcinoma, apresentando elevada eficácia e baixa citotoxicidade.

Abstract

Urothelial carcinoma of the bladder (UCB) is a serious health problem with high mortality and morbidity rate due to its heterogeneity associated with clinical behaviour and histogenesis. Current therapeutical protocols are not effective for every group of patients with UCB, either superficial or invasive. Several compounds are in clinical development with new therapeutic targets in bladder cancer namely mTOR inhibitors.

The Mammalian target of rapamycin (mTOR), a downstream mediator in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, plays an important role in the regulation of cell metabolism, growth, proliferation and survival and alterations in this pathway are common of several malignant diseases including bladder cancer.

Altered or aberrant glycosylation is common in several human carcinomas, especially during tumour progression, modifying cellular adhesion and motility, affecting their invasive and metastatic potential. Aberrant glycosylation is responsible for the characteristic carbohydrate expression associated with malignant transformation, being catalyzed by specific glycosyltransferases and glycosidases. STn-cancer-associated sialyl-Tn antigen the product of an abnormal glycosylation. STn is expressed in many human carcinomas like ovarian, gastric, colon and also bladder cancer. The possible existence of expression of STn and mTOR pathway activation in more advanced tumours of the bladder could help in patients stratification as well as to design new strategy therapeutics.

The main goal of the present set of studies was to evaluate the expression of PI3K/AKT/mTOR pathway activation and STn in UBC both in human tumours correlating its expression with patients prognosis.

We found that PI3K/AKT/mTOR upstream and downstream related molecules showed an equal distribution between non-muscle invasive bladder cancer and muscle-invasive bladder cancer (NMIBC and MIBC), not associating with the stage of the disease. PTEN expression was decreased in advanced stage tumours and correlated with worse overall survival. We also found an association with STn expression and muscle invasion which correlated with decrease

survival. This trend was also found in tumours presenting pAkt, pmTOR and/or pS6 in MIBC STn positive groups.

After we evaluated the expression of PI3K/AKT/mTOR pathway activation and STn in cell lines and animal models to understand the effect of the mTOR inhibitors on survival and proliferation of tumoral cells.

In murine chemically induced urothelial tumours we analyzed the impact of sirolimus – an mTOR inhibitor on tumoral progression. We found that the treated mice presented a decreased number of invasive tumours in parallel with decreased expression of pS6 and STn; we describe for the first time that the administration of sirolimus was effective against STn positive cells providing an apparently link between PI3K/AKT/mTOR activation and STn in invasive tumours. Regarding the mTOR inhibitors we also evaluated the effectiveness of the combination of everolimus (RAD001) with cisplatin using bladder cancer cell lines. We found that this combination decreased cell proliferation in a dose dependent manner in comparison to the chemotherapy agent alone namely in NMIBC.

We also evaluated the correlation between the expression of PI3K/AKT/mTOR pathway activation and STn with tumoral progression and cachexia.

Regarding PI3K/AKT/mTOR and bladder cancer-induced skeletal wasting we found that in rats, BBN induced urothelial carcinoma, there was 17% of body weight loss – cachexia; the reduced levels of pAKT, mTOR and p70S6K suggested the suppression of PI3K/AKT/mTOR pathway.

Observing the correlation of STn in bladder tumours with tumour proliferation and clinical relevance we found that 75% of the high grade bladder tumours, expressing STn presented elevated proliferation rates and high risk of recurrence/progression. Thus STn expression correlated with cell invasive capability and was not expressed by the normal urothelial.

Concerning Patient-derived tumour xenografts we studied the tumour model of bladder cancer preserving the original tumour identity. In the evaluation of the expression of tumour markers p53, p63, KI-67, CK20 and STn we found similarities between the primary tumours and the xenografts although p53 and KI-67 levels of expression increased; STn expression even though decreased was preserved in xenografts reinforcing a STn positive xenograft model.

In summary, our results showed the activation PI3K/AKT/mTOR pathway either in NMIBC and MIBC; this is a key pathway in bladder cancer carcinogenesis and mTOR inhibitors are determinant as potential drugs in bladder cancer with high efficacy and low cytotoxicity.

Contents

List of Tables

List of Figures

List of Acronyms

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AKT/PKB	Protein kinase B
ATP	Adenosine triphosphate
BBN	*N*-butyl-*N*-4-hydroxybutyl nitrosamine
BC	Bladder Cancer
BCG	Bacillus Calmette-Guerin
CIS	Carcinoma in situ
CUB	Carcinoma Urotelial da Bexiga
DEPTOR	DEP domain-containing mTOR-interacting protein
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
eIF4E	Eucaryotic translation initiation factor 4E
FDA	Food and Drug Administration
GSTM1	Glutathione S-transferase M1
IRS-1	Insulin receptor substrate 1
ISUP	International Society of Urological Pathology
kDa	kilodalton
MI	Muscle invasive
MIBC	Muscle invasive bladder cancer
mLST8	Mammalian lethal with SEC13 protein 8
mRNA	Messenger ribonucleic acid
mSin1	Mammalian stress activated protein kinase interacting protein 1
mTOR	Mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
MVAC	Methotrexate, vinblastine, adriamycin and cisplatin
NAT 2	N-acetyltransferase 2
NI	Non-invasive
NMI	Non-muscle invasive
NMIBC	Non-muscle invasive bladder cancer
OS	Overall survival

p70S6K	Ribosomal p70 S6 kinase
PDK 1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PK	Protein kinase
PRAS40	Proline rich Akt substrate 40
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PUNLMP	Papillary urothelial neoplasm of low malignant potential
Raptor	Regulatory associated protein of mTOR
Rb	Retinoblastoma
RC	Radical cystectomy
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin insensitive companion of mTOR
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
S6 40S	Ribosomal protein S6
S6K	S6 kinase
SPSS	Statistical Package for the Social Sciences
STn	Cancer-associated sialyl-Tn antigen
TGF-β	Transforming growth factor beta
TNM	Tumour-node-metastases
TP53	Tumour protein p53
TSC 1/2	Tuberous sclerosis complex 1/2
TUR	Transurethral resection
TURBT	Transurethral resection of bladder tumour
UBC	Urothelial bladder carcinoma
UC	Urothelial carcinoma
WHO	World Health Organization

1 Introduction

1.1 Bladder Cancer

Bladder cancer is the second more common cancer of the urinary tract [Williams and Stein, 2004] and the ninth most common cancer diagnosis worldwide [Ferlay *et al*, 2013; Kaufman *et al*, 2009; Ploeg *et al*, 2009]. In the United States of America and Western Europe the bladder cancer histological type, that represents 90% of all, is the urothelial (transitional) carcinoma [Dall'Era *et al*, 2012]. In other regions of the world, namely Middle East and Africa, the squamous cell carcinoma, represents the more frequent histological type of bladder cancer due to the prevalence of *Schistosoma Heamatobium* infections [Jemal *et al*, 2011].

Bladder cancer treatment varies with the age and stage disease of the patient: the high rate of recurrence and the repeated surgical interventions make the treatment one of the most expensive ones of solid tumours [Sievert *et al*, 2009; Jacobs *et al*, 2010].

Thus, bladder cancer is a serious public health problem, either by its prevalence, mortality, impact in the quality of life of families and patients as well as by its social-economic impact [Li *et al*, 2014; Klotz and Brausi, 2015].

1.1.1 Epidemiology and Etiology

UCB is the seventh most common malignancy in men and seventeenth in women [Burger *et al*, 2013]. An estimated number of 430 000 patients worldwide are diagnosed with bladder cancer annually, and more than 165 000 will die from this disease [Ye *et al*, 2014].

It affects primarily individuals of older age having a peak between 50 and 70 years in man. In the word, the rate of bladder cancer is about 3,5 in men to 1 woman [Shariat *et al*, 2010; Burger *et al*, 2013]. Although men are more likely to develop bladder cancer than women, women present with more advanced disease and have worse survival [Fajkovic *et al,* 2011].

Differences in the gender prevalence of bladder cancer may be due to several factors; it was proposed to be a result of different exposure to carcinogens, genetic factors, anatomic, hormonal and even social factors [Shariat *et al*, 2010; Fajkovic *et al*, 2011].

Bladder cancer incidence is variable between regions and countries: in the United States is the sixth most common cancer with an estimated 74,690 new cases to be diagnosed in 2014 [Charlton *et al*, 2014]. In Europe is the fourth more common cancer in men and the eighth cause of death by cancer [Ferlay *et al*, 2010].

The highest and the lowest incidence rates are found in the countries of Europe : the higher in Spain- 41,5 per 100000 habitants in men and 4,8 in woman, and the lowest in Finland – 18,1 in men and 4,3 in woman [Ferlay *et al*, 2010]. In Portugal, in 2008, there was an estimated of 1935 new cases of bladder cancer with 721 deaths [RON, 2008]. According to national oncology records, in 2008, this disease was the fifth most common cancer type in men and tenth in women (Figure 1) [RON, 2008].

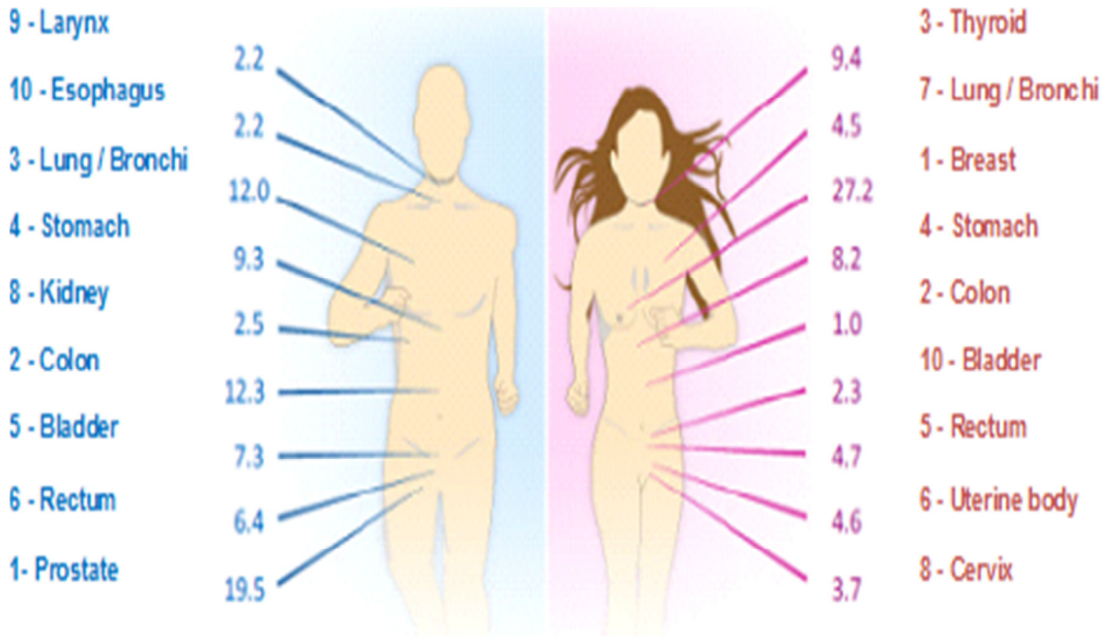

Figure 1: Most frequent tumours by gender, in 2008 (adapted from Roreno, 2008).

Bladder cancer (BC) is a disease of multifactor a aetiology, associated genetic susceptibility and gene–environment interactions, in which cigarette smoking is a major risk, responsible for half of the new cases in men and one third in woman [Wu *et al*, 2008; Chen *et al*, 2010]. Cigarette

smoke is rich in aromatic amines and hydrocarbons that can form DNA adducts. The incidence of bladder cancer is directly related to the duration of smoking and the number of cigarettes smoked per day [Alberg *et al*, 2007].

An immediate decrease in the risk of bladder cancer was observed in those who stopped smoking. The reduction was about 40% within 1-4 years of quitting smoking and 60% after 25 years of cessation, although ex-smokers are always in risk of developing the disease when compared with non- smoking population [Murta-Nascimento *et al*, 2007].

Occupational exposure to aromatic amines and chlorinated by products is the second most important risk factor for bladder cancer. Work-related cases have accounted for 20-25% of all bladder cancer cases, but this percentage in decreasing with the implementation of safety security hazard measures. This exposure is seen mainly in workers from the industrial area of transformation like dyes, rubbers, textiles, paints and leathers [Letasiová *et al*, 2012; Burger *et al*, 2013]. In the case of aromatic amines the risk of bladder cancer due to occupational exposure is significantly greater after 10 years or more of exposure and the mean latency period usually exceeds 30 years [Weistenhofer *et al* 2008; Harling *et al*, 2010]. The histological type usually associated with occupational exposure, is the urothelial or transitional bladder cancer [Tanaka, 2011].

In areas where chronic urothelium infection by *Schistosoma haematobium* is endemic, like Africa and Middle East , including Israel, Egypt, Syria, Iraqi and Iran, there is a high incidence of squamous cell bladder cancer [Santos *et al*, 2011].

Other chronic urinary tract infections have also been linked to the development of bladder cancer while pelvic radiotherapy and chemotherapy agents are associated with the development of secondary bladder malignancies.

Several dietary factors have been considered to be related to bladder cancer (

Table 1); however, the links remains controversial; one of these is the consume of water containing arsenic and/or chlorinated products although the proper ingestion of fluids can reduce the exposure time to this carcinogens, dilute the urine and increase the urinating rate [Kirkali *et al*, 2005; Tanaka *et al*, 2011; Burger *et al*, 2013]. Age and genetic susceptibility are

also associated risk factors for bladder cancer: this risk increases by two fold in first degree relatives of bladder cancer patients. Although there are many studies that identify genetic susceptibility loci for bladder cancer there are only ten validated, including *N*-acetyltransferase 2 (NAT2) slow acetylator and glutathione S-transferase mu 1 (GSTM1)–null , two risk factors when combined with environment exposure to carcinogenic substances like cigarette smoke [Burger *et al*, 2013].

Table 1: Bladder cancer risk factors (adapted from Mitra and Cote, 2009)

Risk factor	Mechanism of carcinogenesis	Primary cellular process(es) altered	Strength of association
Lifestyle			
Tobacco smoking	Exposure to carcinogens in tobacco smoke, including aromatic amines, hydrocarbons, and tar	Cell-cycle regulation, gene regulation	Strong
Hair dye use	Exposure to aromatic amines	Cell-cycle regulation	Weak
Occupation			
Dyestuff manufacturing	Exposure to aromatic amines and aniline dyes	Cell-cycle regulation, gene regulation	Strong
Rubber manufacturing	Exposure to aromatic amines, aniline, and o-toluidine	Cell-cycle regulation	Strong
Painting	Exposure to aromatic amines and aniline dyes	Cell-cycle regulation, gene regulation	Moderate
Leather processing	Exposure to aromatic amines	Cell-cycle regulation	Moderate
Printing	Exposure to aromatic amines and aniline dyes	Cell-cycle regulation, gene regulation	Weak
Hairdressing	Exposure to aromatic amines from hair dyes and gels	Cell-cycle regulation	Weak
Aluminum smelting	Exposure to polycyclic aromatic hydrocarbons	Cell-cycle regulation	Strong
Asphalt paving	Exposure to polycyclic aromatic hydrocarbons	Cell-cycle regulation	Inadequate
Firefighting	Exposure to aromatic amines and polycyclic aromatic hydrocarbons	Cell-cycle regulation	Weak
Truck driving	Exposure to diesel exhaust	Cell-cycle regulation	Moderate
Diet			
Chlorine and chlorination by-	Direct carcinogenic effect	Unconfirmed	Moderate

products (in drinking water)			
Arsenic (in drinking water)	Direct carcinogenic effect	Cell-cycle regulation, signal transduction, gene regulation	Strong
Coffee	Carcinogenic metabolites from caffeine in the urine	Unconfirmed	Inadequate
Artificial sweeteners	Unknown in humans	Unconfirmed	Inadequate
Drugs and therapies			
Phenacetin, cyclophosphamide, pelvic irradiation	Induction of DNA fragmentation	Gene regulation	Moderate
Urologic pathologies			
Schistosoma haematobium	Exposure to toxins and N-nitrosamines	Gene regulation	Strong
Cystitis or other urinary tract infection	Chronic inflammation	Cell-cycle regulation, cell death, gene regulation	Moderate
Urinary calculi	Chronic inflammation	Cell-cycle regulation, cell death, gene regulation	Weak
Ancestry and genetics			
Family history	Genetic predisposition	Depends on the genetic alteration(s)	Strong
NAT2 polymorphism	Inefficient detoxification of aromatic amines	Gene regulation	Strong
NAT1 polymorphism	Promotion of formation of DNA adducts of aromatic amines	Gene regulation	Inadequate
GSTM1	Inefficient detoxification of carcinogens	Gene regulation	Weak

1.1.2 Symptoms and diagnosis of bladder cancer

The majority of patients diagnosed with BC present painless and visible hematuria. Symptoms of bladder irritation like increased rate of urinary frequency occur mainly in patients with carcinoma *in situ* (CIS). The obstructive symptoms occur when the tumour is near the urethra. In advanced disease, patients present lumbar pain, edema of the lower limbs, pelvic palpable mass, weight loss, and bone pain from distant metastasis, however this symptoms almost never occur without a previous story of hematuria [Sharma *et al*, 2009; Urquidi *et al*, 2012].

The clinical investigation should begin with a careful history, including any history of cigarette smoking or occupational exposures. Patients with urinary symptoms should have a urinalysis with urine microscopy and a urine culture to rule out infection [Sharma *et al*, 2009]. Urine Cytology and also Cystoscopy can be used for diagnosis of bladder cancer [Sharma *et al*, 2009; Latini *et al*, 2010]. Urine cytology is a non-invasive test for the diagnosis of bladder cancer with a high specificity but a low sensitivity mainly in low grade bladder tumours. This method is more specific for high grade tumours or CIS while cistoscopy is effective for papillar tumours diagnosis [Sharma *et al*, 2009; Ye *et al*, 2014].

In the recent years there was a development in noninvasive screening tools for bladder cancer, including tumour associated antigens, proliferative antigens, oncogenes, peptide growth factors and its receptors, adhesion molecules, tumoral angiogenesis markers and its inhibitors and cell cycle regulatory proteins. Clinical routine use of this tests was already aproved by the Food and Drug Administation (FDA), however while the tests present a higher sensibility than cytology, a few have the same specificity (Table 2) [Urquidi *et al*, 2012; Wadhwa *et al*,2012; Ye *et al*, 2014].

Table 2: Urine biomarkers for detection and surveillance of bladder cancer (adapted from Ye et al, 2014)

Test	Markers	Sensitivity*	Specificity*
BladderCheck (point-of-care) and Bladder Cancer Test	NMP22	34 – 95%	55-85%
Cytology	Urothelial cells	30 – 92%	93 – 97%
ImmunoCyt	Urothelial cell and immunostain	55 – 90%	33 – 87%
Urovysion	FISH	51 – 92%	55 – 95%
BTA stat (point-of-care) and BTA-TRAK	Bladder Tumor Antigen	36 – 91%	50 – 90%
ACCU-DX (point-of-care)	FDP	60 – 83%	80 – 86%

1.1.3 Histological classification, stage, and grade of bladder cancer

Bladder cancer can be classified histologically as urothelial or non-urothelial; 90% of the cases are urothelial in developing countries and the remaining 10 % are non-urothelial or undifferentiated (Table 3) [Sharma *et al*,2009; Kaufman *et al*,2009; Vishnu *et al*, 2011].

Table 3: Bladder cancer classification (adapted from Sharma et al, 2009)

Epithelial neoplasms	Small cell carcinoma
Urothelial (transitional cell)	Rare neoplasms
neoplasms (90%)	Basaloid squamous cell
Papilloma	carcinoma
Flat	Carcinoid tumour
Papillary	Lymphoepithelial
Papillary urothelial carcinoma	carcinoma
Low malignant potencial	Melanoma
Low – grade	**Nonepithelial**
High – grade	**(mesenchymal)**
Invasive urothelial carcinoma	**neoplasms (1%)**
Lamina propria invasion	Benign
Muscularis própria (detrusor muscle	Hemangioma
invasion	Leiomyoma
Nonurothelial neoplasms	Lipoma
(9%)	Neurofibroma
Squamous cell carcinoma	Paraganglioma
Verrucous carcinoma	Malignant
Adenocarcinoma	Angiosarcoma
Clear cell	Leiomyosarcoma
Hepatoid	Malignant fibrous
Nonurachal	histiocytoma
Signet ring cell	Osteosarcoma
Urachal	Rhabdomyosarcoma

The staging system used in bladder cancer is called TNM, which stands for tumour, node, metastasis (TNM system; Table 4) [Greene 2002; Edge, 2010]. This system staging is based on the growth of the tumour into the bladder and the deep of invasion. The NMIBC include the urothelium carcinomas of papillar morphology (Ta) flat tumours (Tis, *in situ*) and lamina propria infiltrating lesions (T1). When the tumour invades the lamina propria the stage is classified according with the deep of muscle invasion (T2a, T2b). If the tumour invades the surrounding connective tissue the stage is T3 (T3a, T3b). In the T4 stage the tumour has spread to the adjacent structures of the bladder [Chen *et al*, 2009; Edge, 2010].

Table 4: TNM System - American Joint Committee on cancer for Bladder cancer (adapted from Sharma et al, 2009)

Primary tumor (T)

TX: Primary tumor cannot be assessed
T0: No evidence of primary tumor
Ta: Noninvasive papillary carcinoma
Tis: Carcinoma in situ ("flat tumor")
T1: Tumor invades subepithelial connective tissue
T2: Tumor invades muscle
pT2a: Tumor invades superficial muscle (inner half)
pT2b: Tumor invades deep muscle (outer half)
T3: Tumor invades perivesical tissue
pT3a: Microscopically
pT3b: Macroscopically (extravesical mass)
T4: Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall
T4a: Tumor invades prostate, uterus, vagina
T4b: Tumor invades pelvic wall, abdominal wall

Regional lymph nodes (N)

Regional lymph nodes are those within the true pelvis; all others are distant lymph nodes
NX: Regional lymph nodes cannot be assessed
N0: No regional lymph node metastasis
N1: Metastasis in a single lymph node, 2 cm or less in greatest dimension
N2: Metastasis in single lymph node, more than 2 cm but not more than 5 cm in greatest dimension; or multiple lymph nodes, none more than 5 cm in greatest dimension
N3: Metastasis in lymph node more than 5 cm in greatest dimension

Distant metastasis (M)

MX: Distant metastasis cannot be assessed
M0: No distant metastasis
M1: Distant metastasis

Stage grouping

0a	Ta	N0	M0
0is	Tis	N0	M0
I	T1	N0	M0
II	T2a	N0	M0
	T2b	N0	M0
III	T3a	N0	M0
	T3b	N0	M0
	T4a	N0	M0
IV	T4b	N0	M0
	Any T	N1	M0
	Any T	N2	M0
	Any T	N3	M0
	Any T	Any N	M1

The histological grade is established according to the cellular characteristics being an important risk factor to disease progression in NMIBC [Knowles *and* Hurst, 2015]. Recently, there are used 2 systems of classification: the one from 1973 of the *World Health Organization (WHO)* and the

one from 2004 *from WHO/International Society of Urological Pathology (ISUP)* [Babjuk *et al, 2013*].

The major difference is the classification of the tumours of papillar morphology that are classified as grade 1, 2 and 3 in the old system and in the 2004 classification correspond to an urothelial lesion of low-grade malignant potential, (same as grade 1; *papillary urothelial malignancy of low malignant potential – PUNLMP*), urothelial papillary of low grade and urothelial papillary high grade carcinoma (Figure 2: Stage and Grade of BC. A) Stage of bladder cancer according to TNM system B) Histological Grade classification according the WHO 1973, and the ISUP 2004 (adapted from Knowles and Hurst, 2015) [Babjuk *et al, 2013*].

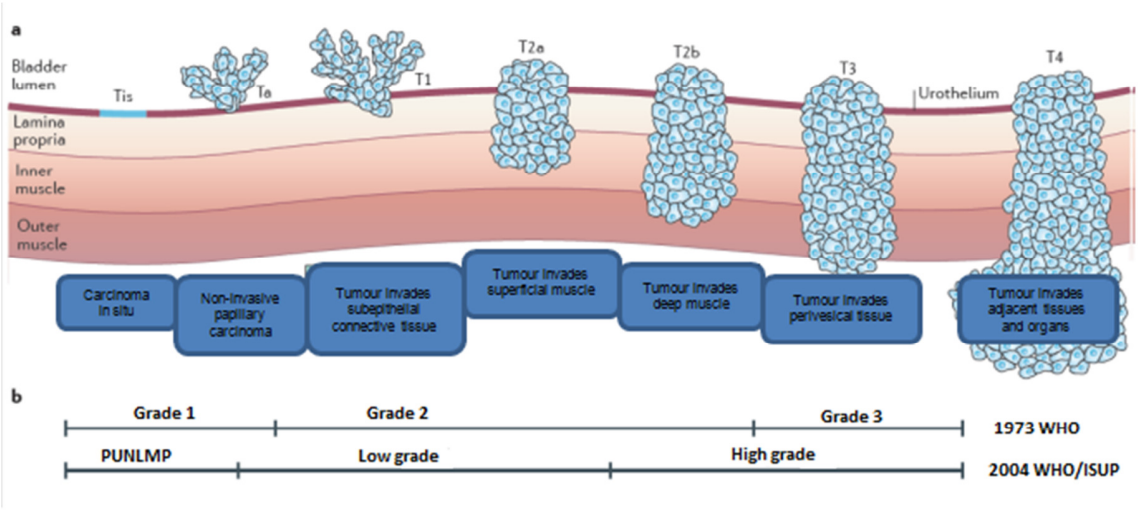

Figure 2: Stage and Grade of BC. A) Stage of bladder cancer according to TNM system B) Histological Grade classification according the WHO 1973, and the ISUP 2004 (adapted from Knowles and Hurst, 2015)

To classify the risk of NMICB the European Association of Urology (EUA) divides the patients in low, medium and high risk of bladder cancer recurrence, that require different treatment approaches. The low risk is defined as a primary tumour, unique, Ta of low grade; medium risk is defined as multiple tumours of low grade; high risk any tumour T1 and/or CIS and multiple recurrent tumours over 3 cm. This stratification is based on clinical and pathological factors, tumour stage and grade, multifocality, size of the tumour, frequency of recurrence after

transurethral resection (TUR), and presence of CIS (Figure 2) [Colombel *et al*, 2008; Bellmunt, 2011; Babjuk *et al*, 2013].

The biologic behaviour and clinical phenotype of NMIBC and MIBC arises from two distinct oncogenic pathways [Tanaka *et al*, 2011]. The low grade tumours always of papillary morphology and generally superficial, or non-muscle-invasive (NMI), represent 70-80% of the BC. This tumours are frequently multifocused with 50-70% recurrence, but normally don't progress and the overall 5 years survival is of 90%. Genetic instability is the key to the accumulation of genetic alterations needed for tumour progression, and in a small percentage (10-15%) these ones can evolve to invasive tumours. It's thought that urothelial hyperplasia is the precursor lesion of this variant. The remaining UBC (about 20%) are diagnosed as high grade, frequently non-papillary, invasive or with metastatic disease. The major reduction seen in overall survival (OS) of these patients, especially due to metastasis, makes these tumours the major responsible by the mortality rates in BC with an OS less than 50%, at 5 years. This variant seems to emerge *de novo* or derives from CIS, and precedes urothelial dysplasia (Figure 3). Many patients that initially present superficial papillary tumours of low grade can develop in the adjacent mucosa CIS [Wu *et al, 2008*; Knowles, 2008; Kaufman *et al*, 2009; Hodges *et al*, 2010; Knowles *et al*, 2014].

The genetic alterations that occur in the NMIBC involve the genes Fibroblast Growth Factor Receptor 3 (FGFR3), Harvey Rat Sarcoma Viral Oncogene Homolog (H-RAS) and PIK3CA [Turo *et al*, 2014; Liu *et al*, 2014]. The other oncogenic pathway of the MIBC presents alterations in several tumoral suppressor genes involved in cell cycle control namely p53, p16 and retinoblastoma (RB) (Figure 3) [Jacobs *et al*, 2010; Netto *et al*, 2013 Turo *et al*, 2015; Liu *et al*, 2014].

The first genetic alterations observed in both oncogenic pathways of BC are in chromosome 9, promoting genetic instability and leading to accumulation of genetic defects. Other chromosomal alterations functional and numerical, associated with BC show some potential both in diagnosis and prognosis namely gains in chromosome 3q, 7p and 17q and deletion of the 9p21 (Figure 3) [Hartmann *et al*, 2002; Netto *et al*, 2013; Knowles and Hurst, 2015].

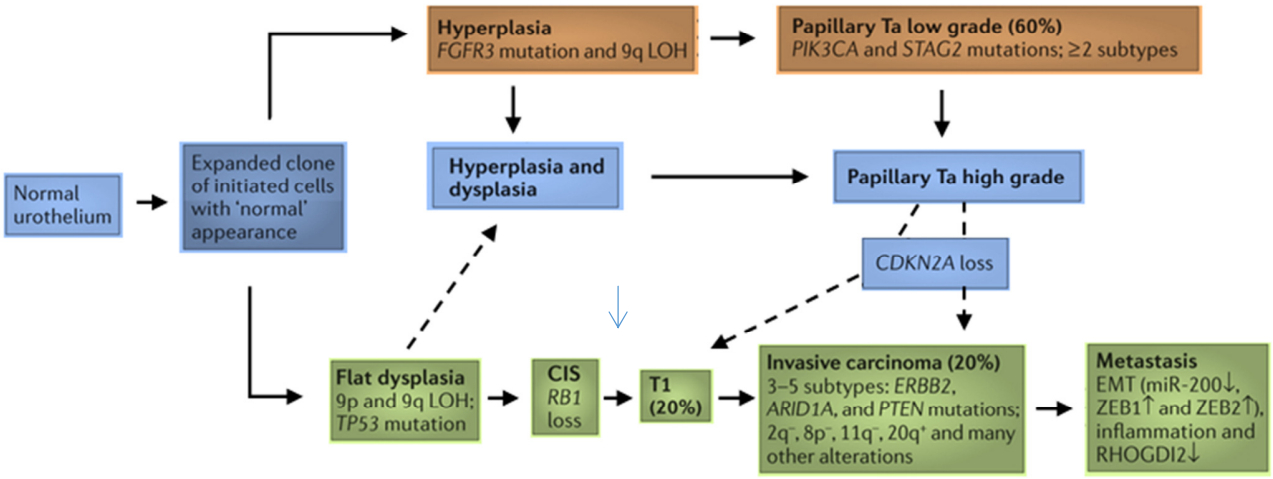

Figure 3: Divergent Oncogenic pathways in bladder cancer. Potential pathogenesis pathways are shown based on histopathological and molecular observations (adapted from Knowles and Hurst, 2015).

1.1.4 Treatment

Bladder cancer have significant heterogeneity whether in pathology or response to therapeutic or survival, driving new strategies of research and selection of the best treatments [Kaufman *et al,* 2009]. Optimal treatment is multidisciplinary, involving urology, pathology, and oncology and after diagnosis a close follow-up is essential [Sharma *et al*, 2009].

Concerning NMIBC (Ta and T1) the usual treatment is a complete eradication of the tumour by TUR including part of the adjacent muscle. A second TUR should be considered when the initial one is incomplete, in tumours of high grade and /or T1 due to the risk of progression by the residual tumour [Babjuk *et al*, 2013]. In this group the problem is the high rate of recurrence and progression to a stage that invades the muscle layer, so it is combined with intravesical adjuvant therapies. The patient selection is based in the associated pathological risks: tumour stage and grade, multifocality, tumour size, presence of CIS and recurrence frequency after TUR [Jacobs *et al*, 2010; Babjuk *et al*, 2013]. The intravesical Bacille Calmette-Guérin (BCG) and

agents like mitomycin and epirrubicine are frequently used from intravesical therapy. It is recommended to all tumours of the bladder with a recent diagnosis a single intravesical instillation in the first 24h after the TUR [Griffiths *et al,* 2012]. The BCG therapy is applied in patients with CIS and recurrent superficial tumours, and the answer to this treatment with BCG or chemotherapy is an important prognostic factor in progression and death caused by BC [Sharma *et al*, 2009; Babjuck *et al*, 2013]. The TUR followed by intravesical BCG once a week during 6 weeks is recommended for CIS [Sharma *et al*, 2009].

Radical cystectomy (RC) should be considered in the NMIBC of high risk like recurrent tumours, T1 of high grade or CIS, in the BCG refractory tumours or in tumours of large size difficult to remove by TUR [Cheung *et al*, 2013]. Although BCG treatment has secondary effects its efficacy in BC recurrence prevention is higher than other chemotherapies [Jacobs *et al*, 2010]. In this group of tumours is difficult to define a treatment to apply in patients with high risk of recurrence and progression to advanced disease stages [Babjuck *et al*, 2013; Kaufman *et al*, 2009; Kulkarni *et al*, 2007].

The MIBC, as referred above is an aggressive carcinoma with a bad prognostic and the main cause of morbidity and mortality by BC. The standard treatment for this BC group is RC with pelvic lymphadenectomy. In men RC involves cystoprostatectomy and cystectomy, usually with hysterectomy in women, followed by a urinary diversion procedure. Patients with metastatic disease are also treated with chemotherapy [Sharma *et al,* 2009; Jacobs *et al*, 2010].

It is expected that the patients receiving adjuvant chemotherapy have 14 months of mean survival expectancy while patients without treatment only have 8 months. About half of the patients subjected to RC are going to recur and die of metastatic disease [Youssef *et al*, 2009; Guancial *et al*, 2015].

In the last 30 years, standard treatment is neoadjuvant and adjuvant chemotherapy combining gemcitabine and cisplatin. Cisplatin is a cytotoxic agent vastly used in BC, with an answer rate of 50-70% although with no recurrence OS of 8 months. In patients with extensive comorbid diseases, they have the carboplatin, which shows a global decreased response compared with

cisplatin [Teply and Kim, 2014]. Carboplatin-based regimens are well tolerated and used in patients with insufficient renal reserve [Sharma *et al*, 2009; Teply and Kim, 2014].

Combination of gemcitabine and cisplatin is the standard treatment for most patients because of its lower toxicity. Combination methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) has comparable effectiveness to gemcitabine/cisplatin; however, toxicity limits its use [Jacobs *et al*, 2010].

There is no therapeutic agents of second line FDA approved, although in Europe vinflunine, an alcaloide, is approved, remaining the only option for second line chemotherapy in metastatic patients after cisplatin based treatment [Gallagher *et al*, 2008; Guancial *et al*, 2015]. Thus, BC is a chemotherapy sensitive carcinoma the OS of a patient with metastatic disease is approximately 15 months [Milowsky *et al*, 2013].

It is urgent to stratify the patients, improving the actual treatment options, and also to develop new therapeutic targets to prevent or delay disease progression, improving the life quality of these patients and survival. *Figure 4* summarizes the symptoms, diagnosis and treatment of BC.

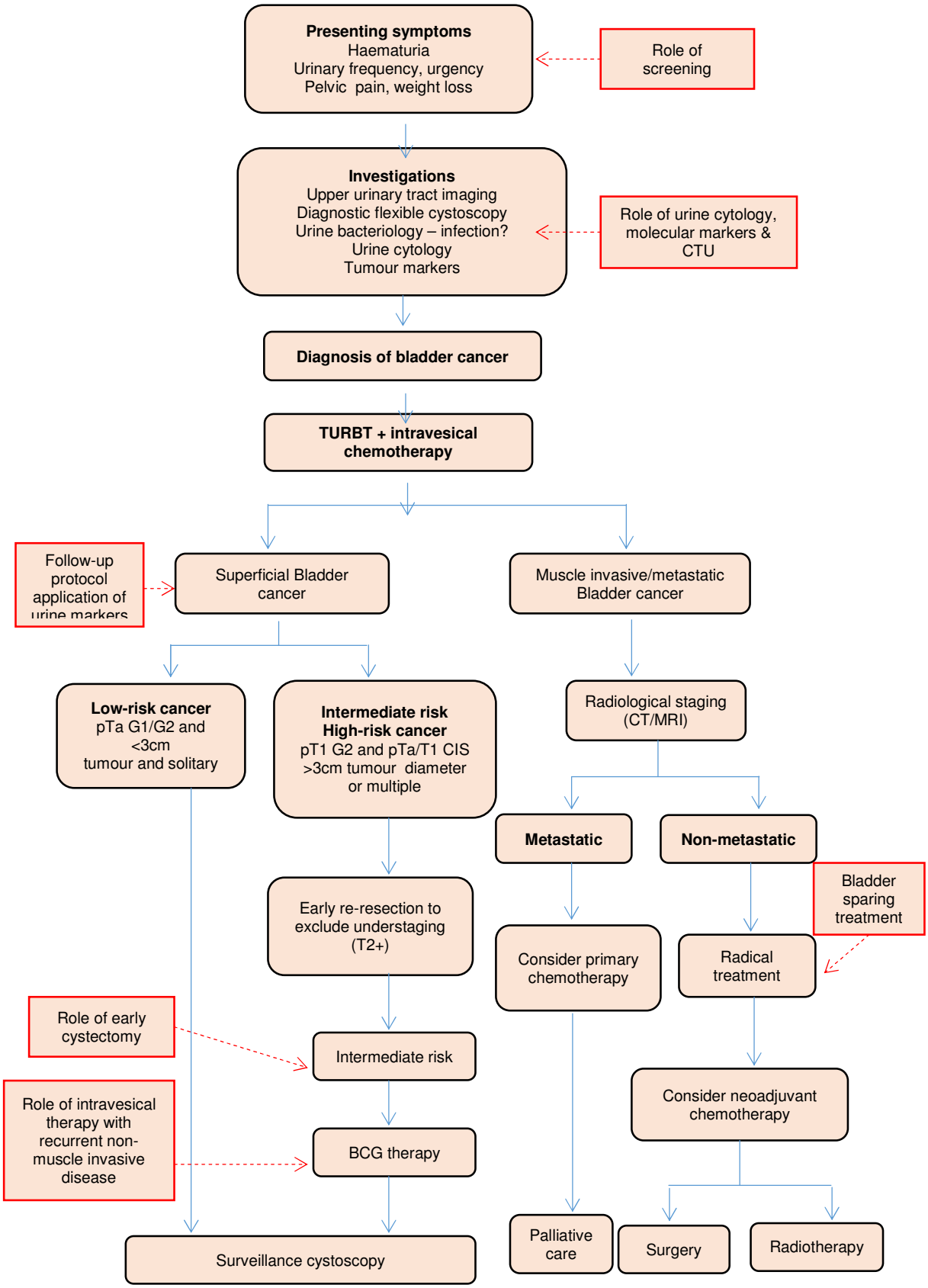

Figure 4: Symptoms, diagnosis and management of BC. Red boxes represent areas of ongoing investigation and clinical trials (adapted Cross and Whelan, 2010).

1.2 Bladder cancer and skeletal muscle wasting

Weight loss in cancer patient is very common especially in advanced stages being responsible for 25-30% of cancer related deaths [Bonetto *et al,* 2011]. Cancer caquexia is associated with a reduction in treatment tolerance, response to therapy, quality of life and survival [Jonhs *et al*, 2014]. The incidence and severity of caquexia is dependent of the tumour type, site and mass and also of the reduced food intake [Tisdale, 1997]. Such abnormal metabolism varies according to the individual and the stage of the disease, also specific effects of the tumour like bowel obstruction and existing morbidities leads to the development of caquexia. There are several mechanisms underlying tumor-host interaction resulting in the abnormal metabolism such as activation of proteolytic pathways preceding pro-inflammatory cytokines production and more recently myostatin expression – a member of the TGF-β. Intracellular signaling pathways leading to myostatin expression are not fully understood. Myostatin increases muscle protein degradation through the proteolytic ubiquitin system and others. Protein degradation is mediated by complex systems namely by PI3K/AKT pathway [McFarlane *et al*, 2006].

Patients with gastric or pancreatic cancer have the highest frequency of weight loss [Tisdale, 2009]. There is a need for the better understanding of the process underlying cancer-induced skeletal muscle wasting as a key point to improve the therapeutic strategies [Padrão *et al*, 2013].

1.3 mTOR Signalization pathway

The Mammalian Target of Rapamycin (mTOR), a downstream mediator in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, plays an important role in the regulation of cell metabolism, growth, proliferation and survival. In particular mTOR is a key protein kinase by monitoring nutrient availability, cellular energy levels, oxygen levels, and mitogenic signals [Liu *et al*, 2009; Laplante and Sabatini, 2009]. Signaling through mTOR is activated by amino acids, insulin, and growth factors, and impaired by nutrient or energy deficiency. In normal cells mTOR plays a key role in cell physiology, homeostasis and regulates numerous components involved in protein synthesis, including initiation and elongation factors, and the biogenesis of

ribosomes [Bjornsti and Houghton, 2004; Dutcher, 2004]. In several diseases and mainly in cancer, this capacity is lost; mutations and continuous activation of this pathway leads to a persistent proliferation and tumour growth [Cheng *et al*, 2013]. Aberrant activation of the mTOR pathway has been widely implicated in many cancers, and increased activity of this pathway is often associated with resistance to cancer therapies, for this reasons it is a promising target for anti-cancer therapies [Fasolo and Sessa, 2012]. This fact was the basis to the development of new targeted therapies, and several have progressed to clinical trials with some success. In recent studies combinational strategies have emerged to overcome resistance to mTOR targeting and enhance their efficacy [Guancial and Rosenberg, 2015]. In several cancer types like renal cell carcinoma and breast cancer rapamycin analogs (rapalogs) have shown some clinical efficacy, although we need more studies exploring the antitumor activity of mTOR targeting-drugs [Motzer *et al*, 2008; Amato *et al, 2013*; Vicier *et al*, 2014]. Besides the limitations of rapamycin-based therapies in the clinical setting led to the development of a second generation of anti-mTOR drugs with substantial improvement of anti-tumor activity both in vitro and in animal-models [Moscheta *et al,* 2014].

1.3.1 The mTOR pathway

mTOR is the target of rapamycin, a macrolide produced by Streptomyces Hygroscopius bacteria and known by its anti-proliferative properties [Cafferkey *et al*, 1993; Sabatini *et al*, 1994]. In the early 1990s, the mTOR was identified and cloned shortly after the discovery of the two yeast genes TOR1 and TOR2, in budding yeast Saccharomyces cerevisiae during a screen for resistance and toxicity of rapamycin [Sabatini *et al*, 1994; Sabers *et al*, 1995]. To date, every eukaryote genome examined including mammals contains a TOR gene [Bjornsti and Houghton, 2004; Pópulo *et al*, 2012].

FRAP (FKBP12-rapamycin-associated protein), RAFT1 (rapamycin and FKBP12 target), RAPT 1 (rapamycin target 1), or SEP (sirolimus effector protein), also known as mTOR, is a serine/threonine kinase of 289 KDa that belongs to the PI3K-related protein Kinase (PIKKs) family, since its C-terminus shares strong homology to the catalytic domain of PI3K [Bjornsti and Houghton, 2004; Vignot *et al*, 2005]. There are two tandem repeated HEAT motifs (protein

interaction domains in the N-terminus of mTOR found in Huntington, Elongation factor 3, PR65/A and TOR), followed by a FAT domain (domain shared by FRAP, Ataxia telangiectasia mutated, and TRRAP. These factors are PIKK family members), a FRB domain (FKBP12-rapamycin-binding site, found in all eukaryotic TOR orthologs), a PtdIns 3-kinase related catalytic domain, an auto-inhibitory domain (repressor domain or RD domain), and a FATC domain (FAT C terminus)- this last one located at the C-terminus of the protein. The FRB domain forms a deep hydrophobic cleft that serves as the high-affinity binding site for the inhibitory complex FKBP12-rapamycin (Figure 5) [Hay and Sonenberg, 2004; Yang *et al*, 2007; Martelli *et al* 2010].

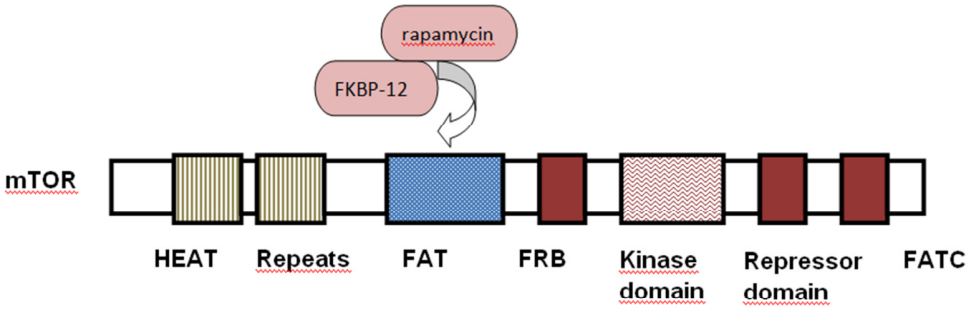

Figure 5: A schematic presentation of mTOR domain structure. (Adapted from Pópulo et al, 2012)

mTOR assembles into two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) with different sensitivities to rapamycin as well as upstream inputs and downstream outputs [Zoncu *et al* 2011; Laplante and Sabatini, 2012].

The mTORC1 is sensitive and mTORC2 is considered resistant to rapamicin. However, Sarbassov and colleagues, shown that long-term treatment with rapamycin can disrupt mTORC2 assembly and function by sequestering newly synthesized mTOR molecules [Sarbassov *et al* 2006].

mTORC1 is composed of mTOR, raptor, mLST8, and two negative regulators, PRAS40 and DEPTOR [Efeyan *et al*, 2012; Lamming *et al*, 2013].

Raptor is a protein of 150Kda with a terminal amino region highly conserved followed by several HEAT repeats and seven carboxyterminal WD40 repeats. A knockout of raptor indicates that it functions in mTOR signaling acting as an adaptor to recruit substrates to mTOR. Alternatively other studies show that raptor negatively regulates mTOR when bound to the kinase. So it remains controversial if raptor is a positive or negative regulator of mtor, though is indispensable for mTOR phosphorilation of S6K1 and 4EBP1proteins [Yang and Guan, 2007; Chiong and Esuvaranathan, 2010].

mLST8 is a 36KDa protein identified after raptor and it contains seven WD40 repeats. Knockout studies both in mammals and yeast suggest that mLST8 regulates positively mTORC1 signaling; other authors also suggest a role of mLST8 in mTORC2. It is believed that this protein is necessary for the full catalytic activity of TOR [Guertin and sabatini, 2007; Yang and Guan, 2007; Zoncu *et al*, 2011].

PRAS40 a proline-rich Akt substrate of 40kDa is a very important component of mTORC1 inhibiting its activity via RAPTOR; thus it links the Akt and the mTOR pathways [Guertin and Sabatini, 2007].

The protein DEPTOR, with 48 kDa, was recently described and it binds to mTOR inhibiting this kinase within TORC1 and TORC2 complexes [Peterson *et al*, 2009; Laplante and Sabatini, 2012].

The mTORC2 comprises six different proteins, several of which are common to mTORC1 and mTORC2: mTOR; rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein (mSIN1); protein observed with Rictor-1 (Protor-1); mLST8; and Deptor [Efeyan *et al*, 2012; Lamming *et al*, 2013].

Rictor is a large protein of 200 KDa unique to mTORC2 Knockdown of rictor results in loss of actin polymerization and cell spreading [Chiong and Esuvaranathan, 2010].

There is some evidence that mSIN1 and Rictor stabilize each other, establishing the structural foundation of mTORC2. Rictor also interacts with Protor-1, but the physiological function of this interaction is not clear. mLST8 is essential for mTORC2 function and Deptor negatively regulates mTORC2 activity [Sengupta *et al*, 2010; Huang and Fingar, 2014].

mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors allowing cell growth by inducing and inhibiting anabolic and catabolic processes, respectively, and also drives cell-cycle progression. mTORC2 responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton, it is insensitive to acute rapamycin treatment but chronic exposure to the drug can disrupt its structure (Figure 6) [Wullschleger *et al*, 2006; Laplante and Sabatini, 2012].

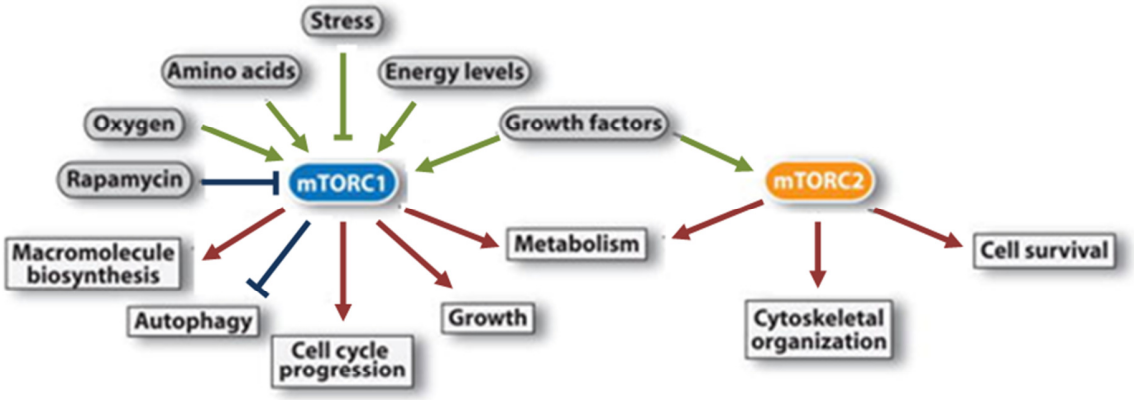

Figure 6: mTORC1 and mTORC2 cross regulation and cell dynamics (adapated from Laplante and abatini, 2012).

1.3.2 Upstream and downstream mTOR pathway regulation

mTOR is only one part of the larger phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway. This pathway is activated, upstream, by growth factors, such as insulin, mitogens, hormones or nutrients, that activate PI3K in the cell membrane. PI3K is a heterodimer that consists of a catalytic subunit (p110) and a regulatory subunit (p85, p55, or p50) [Porta *et al*, 2014, Zarogoulidis *et al*, 2014]. The activated p110 catalytic subunit, encoded by the PIK3CA gene, phosphorylates phosphatidylinositol bisphosphate 4, 5 on the inner leaflet of the cytoplasmic membrane to generate phosphatidylinositol triphosphate (PIP3). The mTOR and this lipid products are involved in a number of cellular processes including cell proliferation, survival, cytoskeletal reorganization, membrane trafficking, cell adhesion, motility, angiogenesis and insulin action [Willems *et al*, 2012]. The binding of insulin to the cell surface receptor causes the

insulin receptor activation with recruitment of insulin receptor substrate 1 (IRS1) and the production of PIP3 though the activation of PI3K [Sarbassov *et al,* 2005].

PIP3 recruits Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK-1) and mTORC2 [Hay and Sonenberg, 2004; Sarbassov *et al,* 2005].

The best studied downstream substrate of AKT is the serine/treonine Kinase mTOR. AKT can directly phosphorylate and activate mTOR, as well as cause indirect activation of mTOR by phosphorylating and inactivating TSC2 (tuberous sclerosis complex 2), which normally inhibits mTOR through the GTP-binding protein Rheb (Ras homolog enriched in brain). When TSC2 is inactivated by phosphorylation, the GTPase Rheb is maintained in its GTP-bound state, allowing for increased activation of mTOR [Hay, 2005].

Activated mTOR can subsequently phosphorylate downstream effectors S6K1 (p70 ribossomal protein S6 Kinase 1) and 4EBP1 (eIF4E binding protein) [Ching and Hansel, 2010]. On clinically healthy conditions, S6K1 and 4EBP1 are bound to eIF3 (eukaryotic initiation factor 3) remaining inactive. With growth stimulations, mTOR binds to eIF3 and phosphorylates S6K1 and 4EBP1. The phosphorylation of S6K1 releases it from eIF3 and activates the kinase. S6K1 activation promotes translation of mRNAs and growth by phosphorylating cellular substrates, such as S6 [Bjornsti and Houghton, 2004; Pópulo *et al*, 2012]. When 4EBP1 binds to translation initiator eIF4E (eukaryotic translation initiation factor 4E) it inhibits cap-dependent mRNA translation. mTOR phosphorylates 4EBP1, and the phosphorylation of 4EBP1 frees it from eIF4E, relieving its inhibitory effect and stimulating translation initiation. Active mTOR enhances cell growth by promoting protein translation and increasing cell mass [Bjornsti and Houghton, 2004; Inoki *et al*, 2005; Sarbassov *et al*, 2005].

The inhibition of mTOR by rapamycin also causes 4EBP1 dephophosrilation which prevents protein translation. There is evidence of a negative feedback loop from the mTOR-S6K1 pathway to the upstream IRS pathway. Activation of mTORC1 and S6K1 regulates IRS-1 leading to a negative feedback regulation of both PI3K and MAPK signaling pathways. In some tumours activation of mTORC1 may be PI3K independent [Moschetta *et al*, 2014].

mTORC2 upstream regulates are less known although studies show that direct association with ribossome is required for mTORC2 activation – event in the dependence of PI3K activity [Carracedo *et al*, 2008]. This mTORC2 upstream regulatory mechanism shows the link between the two mTOR complexes: mTORC2 promotes mTORC1 activity via the AKT-TSC1/TSC2 pathway and in return mTORC1 controls mTORC2 activity through of ribossomal biogenesis [willems *et al* 2012].

The central negative regulator of PI3K/AKT/mTOR signaling cascade is phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which was first discovered in 1997 as a phosphatase, that is mutated or lost in several cancers [Liaw *et al* 1997]. Located on chromosome 10q23, PTEN is a multifunctional phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP3), which is a second messenger molecule produced following class I phosphoinositide 3-kinases (PI3K) activation in response to various growth factors [Sansal and Sellers, 2004]. Cytoplasmic PTEN negatively regulates this pathway by dephosphorylating PIP3 at its D3 position, thereby inhibiting downstream kinase activation and preventing cancer cell growth and survival. In contrast, nuclear PTEN exhibits phosphatase-independent tumor suppressive functions, including regulation of chromosome stability, DNA repair, and apoptosis. The loss of PTEN is implicated in the loss of integrity of the genome [Ching and Hansel, 2010].

1.3.3 **Bladder Cancer and** the mTOR pathway

As stated before the mTOR pathway is determinant for cell growth and proliferation and disruption of this pathway can result in altered cell dynamics and tumour development. Alterations in this pathway are common of several malignant diseases including melanoma, prostate cancer, renal cell cancer, breast cancer and others. Increased activity of this pathway can potentially promote tumorigenesis.

There is evidence that elements in this pathway play an important role in this oncogenesis of bladder cancer, although there is little and inconsistent results about mTOR pathway activation in bladder tumour tissue; previous studies were performed using cell culture or animal models

but its clinical relevance remains uncertain. Previous studies of Hansel and colleagues showed 74% expression of pmTOR in MIBC and a significant association with increased pathological stages and reduced disease-specific survival [Hansel *et al,* 2010]. Also Makhlin *et al* (2011) found pmTOR expression increased in malignant versus normal urothelium – 32% of tumours but with no association with clinical pathological stage. On the other hand there are other studies that showed that expression of mTOR is not increased when comparing with benign urothelium [Park *et al,* 2011]. This pattern of mTOR expression was also described in studies of Afonso and colleagues: 20% of tumour samples score positive for pmTOR expression when comparing with normal urothelium [Afonso *et al*, 2014]. mTOR expression was frequently observed but without stronger intensity in MIBC. Most of these studies are focused on patient with muscle invasive disease.

Other molecular alterations of PI3K/AKT/mTOR pathway effectors have been reported in UBC including PTEN deletions, PTEN mutations, TSC1 inactivating mutation, and activations mutations of PIK3CA. Mutations in PIK3CA gene associate with increased mTOR signaling and bladder cancer cells resistance to apoptosis [Ching and Hansel, 2010]. Also PI3K pathway inhibition reduces the invasive capacity of bladder cancer. Several studies show a decrease or loss in PTEN expression which is associated with aggressive tumor growth, metastases and worsened patient outcomes. In superficial tumours PTEN loss is about 6, 6%, whereas in invasive tumours goes up to 30% [Wu *et al*, 2004; Ching and Hansel, 2010]. Chaux and colleagues when studying superficial tumors observed high levels of PTEN expression associated with higher rates of tumor recurrence, tumor progression and systemic metastasis [Chaux *et al*, 2013].

Regarding pAKT expression, in more than half of UBC its higher expression suggested to contribute to invasion [Wu *et al*, 2004]. Also, there are different staining patterns (nuclear and cytoplasmic) in pAKT expression. Although AKT and pAKT levels are not significantly associated with tumor grade or stage [Sun *et al*, 2011].

In MIBC mTOR pathway is commonly activated though PI3K mutations and inactivation of PTEN and TSC1: 21% demonstrate PI3K mutations, 30% demonstrate evidence of PTEN inactivation.

Also 16% of patients have inactivating mutation in TSC1 [Ching and Hansel, 2010; Kompier *et al*, 2010]. pS6 expression was observed in 55% of this BC cancer group [Hansel *et al*, 2010].

1.3.4 mTOR pathway inhibitors in cancer therapy

The mTOR inhibitors are divided in two groups: rapamycin/rapamycin analogs ou rapalogs and mTOR Kinase inhibitors [Macarulla *et al*, 2009]. Rapamycin, an immunosupressive drug also named sirolimus is a natural compound approved by FDA in 1999 for transplant rejection prevention [Huang and Houghton 2001; Fantus and Thomson, 2015]. Later studies found that its anti-proliferative properties could be used as a key agent in different tumour types, such as small cell lung cancer, breast, pancreatic, prostate cancer, osteossarcoma and B-cell-lymphoma [Muthukkumar *et al,* 1995; Seufferlein and Rozengurt, 1996; Grewe *et al*, 1999; Pang and Faber, 2001; van der Poel *et al*, 2003]. Nowadays, due to limitations in the solubility and pharmacokinetic properties of rapamycin several derivatives have emerged including rapalogs temsirolimus, everolimus and deferolimus. The rapalogs either alone or combined are currently being evaluated in clinical trials as bladder cancer treatments [Zaytseva *et al*, 2012; Moschetta, 2014].

The second generation of mTOR inhibitors are known as ATP-Competitive mTOR kinase inhibitors (TKIs). TKI targets the mTOR kinase domain inhibiting the catalytic activity of mTORC1 and mTORC2 with the advantage of minimizing the feed-back activation of PI3K/AKT, example Torin 1, PP30, PP242, Ku-0063794, AZD8055 and WYE-354 [Zaytseva *et al*, 2012].

Also dual PI3K/mTOR inhibitors are being used in clinical trials including NVP-BEZ 235, XL-735 and PI-103 (Figura 7) [Zask *et al*, 2011; Zaytseva *et al*, 2012].

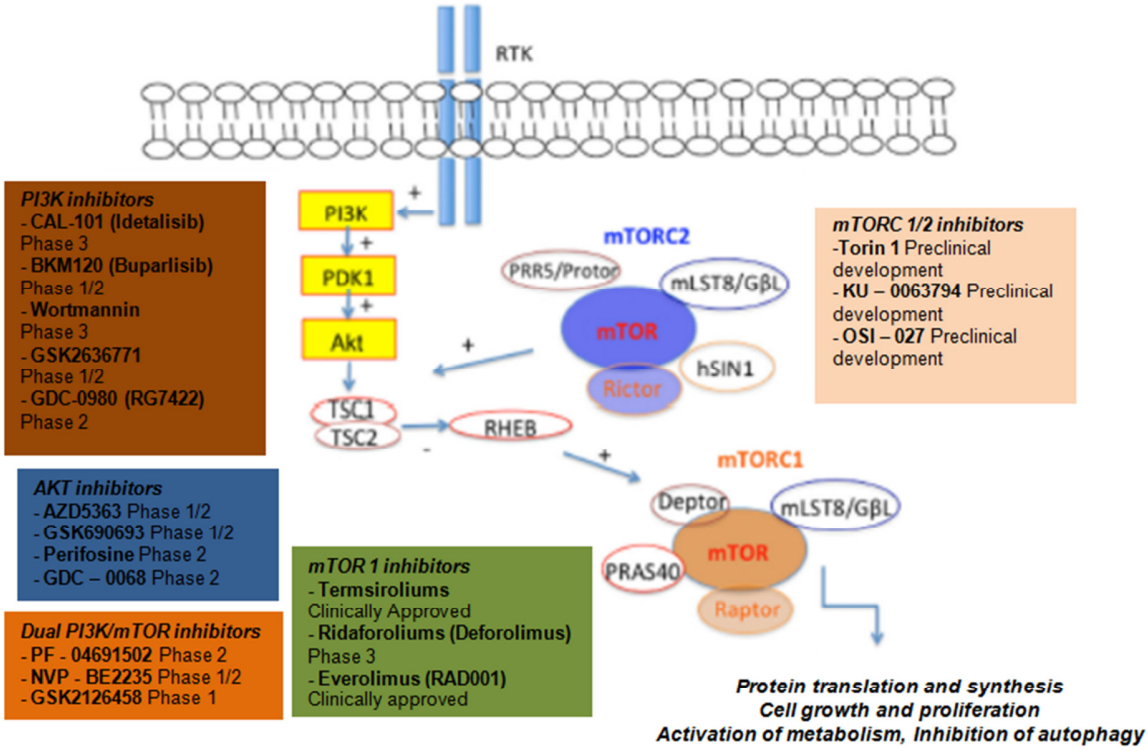

Figure 7: The mTOR pathway as a therapeutic target and the ongoing clinical studies (adapted from Mochetta et al, 2014).

Several compounds referred before are in clinical development as emerging therapeutic targets in bladder cancer. Everolimus has been investigated in a phase II study in pretreated metastatic urothelial tumors. The primary endpoint of two month progression-free survival wasn´t met by everolimus [Milowsky *et al*, 2013]. Another trials with everolimus/temsirolimus in a similar patient population showed a limited efficacy with single-agent [Seront *et al*, 2012; Gerullis *et al*, 2012]. There is in course a phase I/II trial of everolimus and intravesical gemcitabine in BCG-refractory primary or secondary CIS of the bladder (NCT01259063). Another phase two trial is in course combining paclitaxel and cisplatin ineligible patient with advanced urothelial carcinoma (NCT0125136).

Also there was a phase II trial of temsirolimus for patients with advanced bladder cancer with limited efficacy so others trails are in course combining tensirolimus or everolimus and gemcitabine/cisplatin. The dual mTOR inhibitors are also being investigated with promising

preclinical results showing synergistic anti-tumor effects with lapatinib [Becker *et al*, 2014]. The list ongoing trials of mTOR and PI3K inhibitor are reported in table 5.

Table 5: Novel molecularly targeted agents for the PI3K/mTOR under evaluation in patients with bladder cancer

Agent	Description	Trial ID number	Phase	Design
Everolimus	mTORC1 inhibitor	NCT01259063	I/II	Everolimus and intravesical gemcitabine in BCG-refractory primary or secondary carcinoma in situ of the bladder
Everolimus	mTORC1 inhibitor	NCT01215136	II	In combination with paclitaxel for cisplatin-ineligible patients with advanced urothelial carcinoma
Temsirolimus	mTORC1 inhibitor	NCT01827943	II	Second-line therapy for patients with advanced bladder cancer
MLN0128	mTORC1/mTORC2	NCT01058707	I	Oral chemotherapy of, MLN0128 in patients with recurrence or progression of urothelial cancer that is metastatic.
NVP-BEZ235	PI3K/mTOR		Preclinical	
PF-04691502	PI3K/mTOR		Preclinical	
Buparlisib	Pan-classI selective PI3K inhibitor	NCT01551030	II	Patients with metastatic TCC

1.4 Cancer-associated sialyl-Tn antigen (STn)

The sialyl-Tn (STn) antigen is a tumor associated carbohydrate antigen expressed by several human carcinomas and rarely expressed in normal tissues [Cao *et al* 1996]. STn is a short *O*-glycan containing a sialic acid residue α2, 6-linked to GalNAcα-*O*-Ser/Thr. The biosynthesis of STn is mediated by a specific sialyltransferase termed ST6GalNAc I, which competes with *O*-glycans elongating glycosyltransferases and prevents cancer cells from exhibiting longer *O*-glycans [Julien *et al*, 2012].

Several studies show that aberrant glycosylation is a key point in cancer development and metastization [Reis *et al*, 2010]. There is an important role of O-glycans mainly in the attachment and invasion of cancer cells, thus changes in these molecules are determinant for cancer development [Brockhausen, 1999].

STn expression by cancer cells is associated with a poor prognostic and a decreased OS of the patients [Werther *et al*, 1996].

The main focus of research until the last decade was the STn use as a marker for diagnosis and subsequently prognosis in cancer. The recent focus was the targeting of STn by immunotherapy strategies to treat cancers [Julien *et al*, 2012]. An example is the vaccine consisting of STn antigen epitopes to immunize breast cancer patients [Julien *et al*, 2001].

STn is expressed in many human carcinomas like breast, ovarian, gastric, colon and also bladder cancer. In bladder tumours, it is mainly present in advanced stage cases, while absent from most low-grade superficial lesions [Ferreira *et al*, 2013]. Moreover, it is not expressed by the healthy urothelium, denoting a cancer-specific nature and favoring disease dissemination [Ferreira *et al*, 2013; Carrascal *et al*, 2014].

STn antigen is frequently observed in areas of invasion of the basal and muscle layers, suggesting its association with cell migration and invasion. Though STn is part of a malignant bladder cancer phenotype, as previously observed for other carcinomas [Clement *et al*, 2004; Julien *et al*, 2006; Ohno *et al*, 2006; Ozaki *et al*, 2012; Pinho *et al*, 2007].

Based on this information, it should be interesting to evaluate the expression of STn and Tn antigen its precursor, and PI3K/Akt/mTOR pathway activation in bladder cancer.

The possible existence of expression of STn and mTOR pathway activation in more advanced tumours of bladder could help to design a new strategy therapeutic.

1.5 Urinary bladder cancer cell lines and animal models in bladder cancer therapy

Traditional cell lines established from patient tumors and adapted to proliferate in culture have been widely studied. *In vitro* assays are used in the preclinical development of new drugs to discover, validate and evaluate the potential of new agents [Monks *et al*, 1991]. For more than a half century, tumor cell lines served as a foundation for cancer research, they are easy to propagate and study under defined conditions. However, continuous passages and culture of cells *in vitro* selects the cells better adapted to thrive in plastic dishes and excludes the variables associated with tumor stroma and other tumor micro environmental factors [Gillet *et al*, 2013].

Several tumor models have been used in basic science studies and clinical trials to increase our understanding of molecular mechanisms underlying tumor initiation, progression, metastasis and chemoresistance. There has been a continuous search for an ideal model that would mimic the clonal origin of human tumors, their histopathology, the multistage process of carcinogenesis and progression and their clinical behavior.

Animal models have been used in UBC to predict the development and preclinical evaluation of news strategies for bladder cancer treatment [Arentsen *et al*, 2009]. The use of chemically induced urinary bladder cancer models allowed the study of the different stages of carcinogenesis and progression. According to the literature rodents proven to be suitable for these kinds of studies [Palmeira *et al*, 2010]. Rats and mice don´t develop, under normal conditions, spontaneous urinary bladder tumors. The occurrence of non-neoplastic urothelial lesions, such as inflammation and hyperplasia are also uncommon in these species [Oliveira *et al*, 2006].

More than three decades ago a rat model of urothelial carcinogenesis has been described, induced with N-butil-N-(4-hidroxibutil) nitrosamina (BBN) – a genotoxic carcinogen [Becci *et al*, 1981; Oliveira *et al*, 2006]. Mice exposition to BBN in drinking water induces the development of preneoplastic and neoplastic lesions in the urothelium of rodents, namely hyperplasia, dysplasia, low and high-grade papillary UBC, CIS and MIBC [Oliveira *et al*, 2006].

This well-established model reflects the environment in urothelial carcinoma and reproduces tumor-host interaction [Arentsen *et al*, 2009]. The main limitations of this model are the costs and the long experimental protocol.

Patient derived tumor xenograft models (PDTXM) are obtained implanting human derived tumor cell into immunocompromised mice which do not reject human cells. The tumors growing in these animals derive directly from patient tumor samples with minimal manipulation and recapitulate the biological characteristics of the tumor of origin. These models have shown to retain the cellular structure and molecular markers of the original tumors and have high predictive power [Fichtner *et al*, 2004; Rubio-Viqueira and Hidalgo, 2009; Dong *et al*, 2010]. PDTXM are suitable to evaluate individually the effectiveness of anticancer drugs, providing not only an investigational platform but a potential therapeutic decision making tool [Hidalgo *et al*, 2011].

Xenografts models are easy and low cost when compared to the genetic engineered models. PDTXM preserves original cells heterogeneity, tumor phenotype and malignant potential of human tumors. Subcutaneous bladder tumor models have been widely used because of the ease of assessing tumour growth kinetics and because the orthotopic model is technically more difficult [Johnson *et al*, 2001; Rubio-Viqueira and Hidalgo, 2009; Arentsen *et al*, 2009].

The main limitations of this model are the long lag period before tumor growing and low take rate, especially in the first passage. The stroma and blood supply is provided by the host and the tumor is not growing in the organ of origin [Rosfjord *et al*, 2014; Wilding and Bodmer, 2014.

At present the development of new mTOR inhibitors could be accessed in this type of animal model identifying new targets and treatment regimens that improve the OS of bladder cancer patients.

2 Aims of the study

At the initial diagnosis of bladder cancer, 70% of cases are diagnosed as non-muscle-invasive bladder cancer (NMIBC) and approximately 30% as muscle-invasive bladder cancer (MIBC) [Witjes *et al*, 2013]. Regarding NMIBC the problem stands in recurrence and progression, and in the MIBC the high morbidity and mortality. Although this bladder tumours are chemotherapy sensitive, with the current strategies, there are still patients that don`t respond to the treatment. There is urgency in the identification of biomarkers to assist prognostic and the development of more effective targeted therapeutics namely signalling pathways frequently altered and tumour antigen markers.

A. <u>There are evidence that PI3K/Akt/mTOR pathway proteins and STn expression may hold value for bladder cancer patient stratification. With that in mind we performed several studies with the following aims:</u>
   - to evaluate the expression and PI3K/Akt/mTOR pathway activation and STn in bladder cancer at different stages;
   - to correlate PI3K/Akt/mTOR pathway proteins expression (pAkt, pmTOR and pS6 e PTEN) with bladder cancer patient stratification;
   - to evaluate the regulation of PI3K/Akt/mTOR and activation of the ubiquin- proteosome pathways in bladder cancer- induced skeletal muscle wasting;
   - to correlate the regulation of PI3K/Akt/mTOR and the expression of STn in bladder tumours its correlation with tumour proliferation and clinical relevance.

B. <u>Recently it has been reported that mTOR inhibitors could have an important role to play in bladder-cancer treatment and may restore chemosensitivity in resistant tumours</u>
   - to use murine chemically induced urothelial tumours treated with Sirolimus to assess the impact of mTOR inhibitors on cell protein expression;
   - we intend to assess RAD001's in vitro ability to enhance Cisplatin cytotoxicity in three human bladder-cancer cell lines representative of the bladder cancer heterogeneity.

C. <u>Patient-derived tumor xenografts (PDTXs) are said to accurately reflect the heterogeneity of human tumours. In the case of human bladder cancer, few studies are available featuring these models. Patient-derived tumour xenografts (PDTXs) have been shown to be a highly predictive model to test standard chemotherapy and for identification of tumour types that might benefit from new treatments in clinical trials</u>

   - We intend to establish and characterize a direct PDTXs model suitable to study the characteristics and behaviour of human tumours, namely the response to specific chemotherapeutic agents;

- We intend to develop a direct PDTXs non-human model conserving the STn expression, and use it to compare tumour response and resistance to drugs.

3 Scientific articles

3.1 PI3K/Akt/mTOR pathway activation and STn expression in bladder cancer.

3.1.1 Paper I

Abnormal protein glycosylation and activated PI3K/AKT/mTOR pathway: role in bladder cancer prognosis and targeted therapeutics.

Costa C and Pereira S, Lima L, Peixoto A, Fernandes E, Neves D, Neves M, Gaiteiro C, Tavares A, Gil da Costa R, Cruz R, Amaro T, Oliveira P, Ferreira A and Santos L.

Article accepted for publication in the journal *PLOS ONE*

PLOS | ONE

RESEARCH ARTICLE

Abnormal Protein Glycosylation and Activated PI3K/Akt/mTOR Pathway: Role in Bladder Cancer Prognosis and Targeted Therapeutics

Céu Costa[1,2,3☯], Sofia Pereira[1,2,3☯], Luís Lima[1,4,5], Andreia Peixoto[1], Elisabete Fernandes[1], Diogo Neves[1], Manuel Neves[1], Cristiana Gaiteiro[1], Ana Tavares[1,6], Rui M. Gil da Costa[1,7], Ricardo Cruz[8], Teresina Amaro[9], Paula A. Oliveira[10], José Alexandre Ferreira[1,11]*, Lúcio L. Santos[1,3,12]*

1 Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Rua Dr. António Bernardino de Almeida, Porto, Portugal, **2** ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Porto, Portugal, **3** Health Sciences Faculty of University Fernando Pessoa, Porto, Portugal, **4** Nucleo de Investigação e Informação em Farmácia - Centro de Investigação em Saúde e Ambiente (CISA), School of Allied Health Sciences – Polytechnic Institute of Oporto, Porto, Portugal, **5** Institute of Pathology and Molecular Immunology of the University of Porto (IPATIMUP), Porto, Portugal, **6** Department of Pathology, Portuguese Institute of Oncology, Porto, Portugal, **7** Faculty of Engineering, Laboratory for Process, Environment, Biotechnology and Energy Engineering (LEPABE), University of Porto, Porto, Portugal, **8** Department of Urology, Portuguese Institute of Oncology, Porto, Portugal, **9** Department of Urology, Hospital Pedro Hispano, Matosinhos, Portugal, **10** Department of Veterinary Sciences, CITAB, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal, **11** Mass Spectrometry Center of the University of Aveiro, Campus de Santiago, Aveiro, Portugal, **12** Department of Surgical Oncology, Portuguese Institute of Oncology, Porto, Portugal

☯ These authors contributed equally to this work.
* josealexandreferreira@ua.pt (JAF); llarasantos@gmail.com (LLS)

OPEN ACCESS

Citation: Costa C, Pereira S, Lima L, Peixoto A, Fernandes E, Neves D, et al. (2015) Abnormal Protein Glycosylation and Activated PI3K/Akt/mTOR Pathway: Role in Bladder Cancer Prognosis and Targeted Therapeutics. PLoS ONE 10(11): e0141253. doi:10.1371/journal.pone.0141253

Editor: Francisco X. Real, Centro Nacional de Investigaciones Oncológicas (CNIO), SPAIN

Received: July 28, 2015

Accepted: October 6, 2015

Published: November 16, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by Portuguese Foundation for Science and Technology (FCT) Postdoctoral grants SFRH/BPD/66288/2009 (José Alexandre Ferreira), SFRH/BPD/101827/2014 (Luis Lima), SFRH/BPD/85462/2012 (Rui Gil da Costa) and PhD grants SFRH/BD/103571/2014 (Elisabete Fernandes) and SFRH/BD/111242/2015 (Andreia Peixoto). FCT is co-financed by European Social Fund (ESF) under Human Potential Operation Programme (POPH) from National Strategic

Abstract

Muscle invasive bladder cancer (MIBC, stage ≥T2) is generally associated with poor prognosis, constituting the second most common cause of death among genitourinary tumours. Due to high molecular heterogeneity significant variations in the natural history and disease outcome have been observed. This has also delayed the introduction of personalized therapeutics, making advanced stage bladder cancer almost an orphan disease in terms of treatment. Altered protein glycosylation translated by the expression of the sialyl-Tn antigen (STn) and its precursor Tn as well as the activation of the PI3K/Akt/mTOR pathway are cancer-associated events that may hold potential for patient stratification and guided therapy. Therefore, a retrospective design, 96 bladder tumours of different stages (Ta, T1-T4) was screened for STn and phosphorylated forms of Akt (pAkt), mTOR (pmTOR), S6 (pS6) and PTEN, related with the activation of the PI3K/Akt/mTOR pathway. In our series the expression of Tn was residual and was not linked to stage or outcome, while STn was statically higher in MIBC when compared to non-muscle invasive tumours ($p = 0.001$) and associated decreased cancer-specific survival (log rank $p = 0.024$). Conversely, PI3K/Akt/mTOR pathway intermediates showed an equal distribution between non-muscle invasive bladder cancer (NMIBC) and MIBC and did not associate with cancer-specif survival (CSS) in any of

Reference Framework (NSRF). The authors also acknowledge financial support from ICBAS-UP (Céu Costa and Sofia Pereira) and the Portuguese Association of Urology/Pfizer prize 2013. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

these groups. However, the overexpression of pAKT, pmTOR and/or pS6 allowed discriminating STn-positive advanced stage bladder tumours facing worst CSS ($p = 0.027$). Furthermore, multivariate Cox regression analysis revealed that overexpression of PI3K/Akt/mTOR pathway proteins in STn+ MIBC was independently associated with approximately 6-fold risk of death by cancer ($p = 0.039$). Mice bearing advanced stage chemically-induced bladder tumours mimicking the histological and molecular nature of human tumours were then administrated with mTOR-pathway inhibitor sirolimus (rapamycin). This decreased the number of invasive lesions and, concomitantly, the expression of STn and also pS6, the downstream effector of the PI3K/Akt/mTOR pathway. In conclusion, STn was found to be marker of poor prognosis in bladder cancer and, in combination with PI3K/Akt/mTOR pathway evaluation, holds potential to improve the stratification of stage disease. Animal experiments suggest that mTOR pathway inhibition could be a potential therapeutic approach for this specific subtype of MIBC.

Introduction

Bladder cancer is the second most deadly genitourinary tumour and presents significantly worse prognosis upon *muscularis propria* invasion [1]. Approximately 20–30% of the newly diagnosed cases are muscle invasive bladder cancers (MIBC; T2-T4 stages), while 50% are non-muscle invasive bladder tumours (NMIBC) with high potential to progress to invasion. MIBC treatment includes cystectomy and (neo)adjuvant cisplatin-based chemotherapy regimens [2]. However, significant variations in the natural history of the disease and responses to treatment can be observed between tumours with identical histological features, reflecting their high molecular heterogeneity [3]. Furthermore, approximately 50% of cases develop metastasis within 5 years, urging the identification of biomarkers to assist prognostication and the development of more effective targeted therapeutics [4].

To meet this need, we have recently addressed the expression of the cancer-associated sialyl-Tn antigen (STn) on a small prospective series of unselected bladder cancer patients [5]. STn is an abnormal post-translational modification that results from a premature stop in cell-membrane proteins *O*-glycosylation by sialylation of the Tn antigen (Fig 1A). In bladder tumours, STn it was mainly present in advanced stage cases, while absent from most low-grade NMIBC [5]. Moreover, it was not expressed by the normal urothelium, denoting a cancer-specific nature [5]. Studies *in vitro* showed that STn expression endowed bladder cancer cells with high invasion capability [5] and an immunotolerogenic phenotype, potentially favoring disease dissemination [6]. Alterations in cell-surface protein glycosylation have be implicated in the activation of intracellular oncogenic signalling pathways [7], including the phosphoinositide-3 kinase (PI3K)/Akt signalling pathway [8] which is thought to play a critical role in bladder cancer development. These preliminary observations support the hypothesis that STn expression may play a key role in disease outcome, which warrants a deeper investigation. Several studies also suggest that Tn antigen, which is a precursor of STn, may be also implicated in oncogenic events [7]; however nothing is known about the expression of this glycan in bladder tumours.

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) pathways are interconnected signaling cascades essential for bladder cell growth and survival (Fig 1B). The PI3K/Akt/mTOR or mTOR pathway integrates a multiplicity of extracellular signals to regulate downstream signaling and protein synthesis, which ultimately leads to

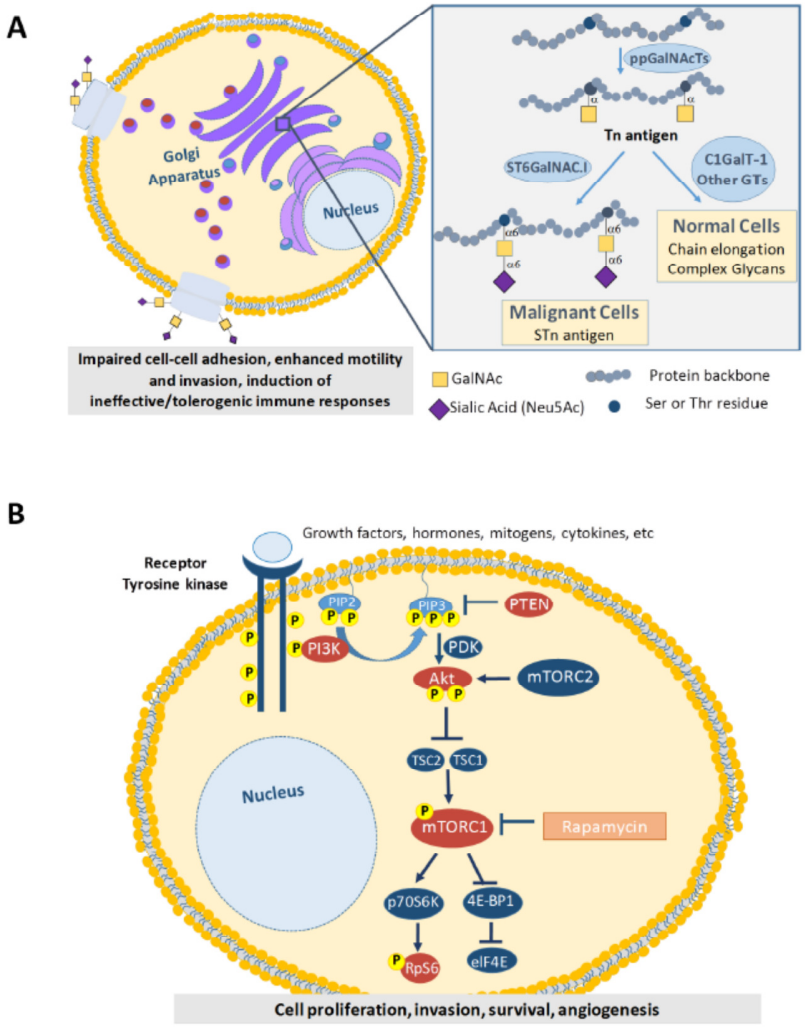

Fig 1. Schematic representation of membrane protein *O*-glycosylation and the PI3K/Akt/mTOR pathways. A) Representation of membrane protein *O*-glycosylation with emphasis on the STn expression by cancer cells. This is a highly regulated process of critical importance for protein stability and function. Briefly, newly synthesized proteins are *O*-glycosylated in the Golgi apparatus by the ppGalNAcTs-mediated addition of GalNAc moiety to Ser/Thr residues. This originates the Tn antigen (GalNAc-*O*-Ser/Thr-protein backbone), which is the simplest O-glycan. In normal cells these chains are extended through the sequential addition of other sugars first by CGALT-1 and then other enzymes. This culminates in highly complex, heterogeneous and elongated glycans often terminated by ABO or Lewis blood group related antigens (left drawing). In cancer cells the Tn antigen is immediately sialylated by ST6GalNAc.I, originating the STn antigen (Neu5Ac-GalNAc-*O*-Ser/Thr-protein backbone), thereby inhibiting further chain elongation (right drawing). The expression of STn at the cell surface influences cell-cell adhesion and cancer cell recognition, favouring motility, invasion and immune escape. B) Schematic representation of the PI3K/Akt/mTOR pathway, which is ubiquitously activated in bladder tumours. This is a highly conserved pathway regulated mainly by a wide variety of extracellular signals, including mitogenic growth factors, hormones, nutrients, cellular energy levels, and stress conditions. These signals activate tyrosine receptor kinases that recruit PI3K, which catalyses the conversion of membrane-bound PIP2 to PIP3. Then Akt and PDK-1 are activated through binding to PIP3. PTEN preferentially dephosphorylates PIP3, inhibiting signalling progression. Full Akt activation requires double phosphorylation by PDK-1 itself and PDK-2 (not shown). Akt phosphorylates mTOR directly or may also inactivate TSC1/TSC2 complex, inhibiting mTOR inactivation. mTORC1 triggers cell growth and proliferation by phosphorylating eukaryotic translation regulators, among these p70S6 kinase (p70S6K or S6K1) that, in turn, phosphorylates the

ribosomal protein S6 (pS6), and the eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1). For the protein mTOR to activate its signalling cascade, it must form the rapamycin-sensitive ternary complex mTORC1. Key PI3K/Akt/mTOR-pathway proteins pAkt, pmTOR and pS6 explored in this studied are highlighted by orange circles.

doi:10.1371/journal.pone.0141253.g001

a competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance [9]. The signaling cascade begins with PI3K activation in the cell membrane followed by serine/threonine kinase Akt cell membrane translocation and activation. The best studied downstream substrate of Akt is the serine/threonine kinase mTOR, whose downstream effector is S6 kinase-1 (S6K1). In particular, a subset of mTOR pathway alterations have been shown to occur in bladder cancer, such as mutations in *PIK3CA* gene, which culminates with increased mTOR signaling and bladder cancer cells resistance to apoptosis [10]. Moreover, the pharmacological or biochemical inhibition of the PI3K pathway drastically reduced the invasive capacity of bladder cancer cell lines. Furthermore, over half of primary human bladder tumours present high Akt phosphorylation and the aberrant activation of this pathway has been suggested to contribute to invasion [11]. Another event influencing mTOR pathway activation in bladder tumours involves the loss of tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) function [12]. PTEN normally suppresses activation of the PI3K/Akt/mTOR pathway antagonizing PI3K and preventing activation of Akt and PDK-1. PTEN also functions to regulate chemotaxis and cell motility, thereby promoting tumor invasion [13]. In summary, there are evidences that a comprehensive evaluation of PI3K/Akt/mTOR pathway associated proteins may hold significant potential for value for patient stratification. Moreover, many preclinical and clinical studies support that mTOR inhibitors, such as sirolimus (rapamycin) and their derivatives may improve cancer treatment [13,14].

Based on these observations we hypothesize that Tn and/or STn may act synergistically with the mTOR pathway to drive bladder cancer progression. As such, we have devoted to evaluating the expression of STn and proteins associated with the activation of the PI3K/Akt/mTOR pathway activation in bladder tumours at different stages. We anticipate that the combination of extracellular and intracellular oncogenic events may improve patient stratification and provide insights for novel therapeutics. Furthermore we have estimated the impact of sirolimus in chemically-induced urothelial tumours in mice, envisaging the creation of a rationale for more effective bladder cancer therapeutics.

Materials and Methods

Ethics Statement

This work involves experiences in tumour samples of patients diagnosed with bladder cancer in the Portuguese Insitute of Oncology of Porto. All procedures were performed after patient's written informed consent and approved by the Ethics Committee of Portuguese Institute of Oncology—Porto. All clinicopathological information was obtained from patients' clinical records.

It also involves animal experiments. All procedures involving animals were performed in accordance with the European Directive 2010/63/EU. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation (Direção Geral de Veterinária, Approval no. 520/000/000/2003). All mice used in the experiment were acclimatized for one week under routine laboratory conditions before starting the experiments. They were housed randomly in groups of 4–5 in plastic cages, with hard wood chips for bedding. The animals were maintained in a room with a controlled

temperature of 23±2°C, a 12-hour light/dark cycle and 55±5% humidity. The animals' drinking solutions were changed once a week or earlier if necessary, and the volume drunk was recorded. Weekly food intake was also noted. All mice were monitored throughout the experiment for signs of distress and loss of body weight. The animals were sacrificed with 0.4% sodium pentobarbital (1 ml/Kg, intraperitoneal).

Population

This study was performed in a retrospective series of 96 formalin-fixed paraffin-embedded bladder tumours obtained from archived paraffin blocks at the Portuguese Institute of Oncology—Porto (IPOP), Portugal. Bladder tumours were extracted from 82 men and 14 women, ranging in age from 38 to 92 years (median of 69.5 years), admitted and treated at the IPOP between 2005 and 2007. Forty seven of the examined tumours were histologically classified as NMBIC (Ta and T1) and 49 as invasive lesions (T2-T4). Sixteen were low grade and 80 were high grade tumours, according to the 2004 WHO grading criteria. Furthermore, carcinoma *in situ* (CIS) was found concomitantly in 20.8% of the patients. The average follow up time period was 45 months (1–134 months). Cystectomy was performed in 64 patients (66.7%) while the other 32 (33.3%) were submitted to transurethral resection. Lymphadenectomy was performed in approximately 47% of the patients and from those 37% presented metastasis. Fifty four (56.3%) tumours were primary and 42 (43.7%) were recurrent tumors. From the recurrent tumours, 38% had no prior treatment, 27% were treated with Mitomicin C, 11% with BCG and 19% were submitted to both treatments. Moreover 5% of these patients were treated with neoadjuvant chemotherapy prior to the cystectomy. Table 1 summarizes the clinicopathological information.

Cancer-specific survival (CSS) was defined as the period between the tumour removal by surgery and either patient death by cancer or the last follow-up information. All procedures were performed after patient's informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients' clinical records.

Immunohistochemistry

The expressions of STn antigen, its precursor Tn, and phosphorylated forms of Akt (pAkt), mTOR (pmTOR), S6 (pS6) and PTEN in bladder tumours were accessed by immunohistochemistry using the avidin/streptavidin peroxidase method, as described by Ferreira et al. [5]. Information on the primary antibodies and dilutions used in this study are summarized in Table 2. Immunoreactivity was revealed using diaminobenzidine (DAB, Thermo Scientific LabVision) as chromogen and sections were counterstained with Harris's hematoxylin. Negative controls were performed by replacing the primary antibody with 5% bovine serum albumin (BSA). Positive controls were known positive tissues for the antigens under study.

Immunohistochemistry scoring of human tumours

The immunostained sections were assessed double-blindly by light microscopy by two independent observers (CC and SP) and validated by an experienced pathologist (TA). Disaccording readings were re-analyzed using a double-headed microscope (Olympus BX46; Olympus Corporation), and consensus was reached. A semi-quantitative approach was established to score the immunohistochemical labeling based on the extent and intensity of the staining.

Given the absence of Tn and STn in the healthy urothelium [5], tumours were classified as positive for these antigens when membrane and/or cytoplasmic immunoreactivity were observed in more than 5% of the tumour, as described by Ferreira et al. [5,15]. pAkt, pmTOR, pS6 and PTEN expressions were scored according to the staining intensity (weak-1 point;

Table 1. Clinical-pathological data of the studied sample (*n* = 96).

Age, *years*		
	median [min—max]	69.5 [38–92]
Gender, *n* (%)		
	Male	82 (85.4%)
	Female	14 (14.6%)
Stage, *n* (%)		
	Ta	27 (28.1%)
	T1	20 (20.8%)
	T2	9 (9.4%)
	T3	20 (20.8%)
	T4	20 (20.8%)
Grade, n (%)		
	Low	16 (16.7%)
	High	80 (83.3%)
Recurrence status, n(%)		
	Primary	54 (56.3%)
	Recurrent	42 (43.7%)
Associated Cis, n(%)		
	No	76 (79.2%)
	Yes	20 (20.8%)
Metastasis, n(%)		
	No	19 (63.3%)
	Yes	11 (36.7%)
Follow-up, *n (%)*		
	Alive, lost or death from other causes	67 (69.8%)
	Death from cancer	29 (30.2%)

doi:10.1371/journal.pone.0141253.t001

moderate-2 points; strong-3 points) multiplied by the percentage of positive cells (0–5%-0 points; >5–25%-1 point; >25–50%-2 points; >50–75%-3 points; >75–100%-4 points). Based on the classification proposed by Nishikawa et al. [16], tumours with a score <6 were considered negative, whereas those with a score≥6 were classified as positive (overexpression). pAkt was evaluated based on nuclear immunoreactivity, pmTOR and pS6 based on cytoplasmic expression and PTEN on both cytoplasmic and nuclear staining, as suggested by other publications [17,18].

Animal experiments with sirolimus and immunohistochemistry scoring

Histological sections of Imprinting Control Region (ICR) mice bearing N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder lesions, resulting from our previous work on the impact of sirolimus on bladder cancer [19], were elected for this study. Briefly, four-week-old male ICR mice (25g; Harlan, Barcelona, Spain) were randomly distributed into four groups, as described in detail in a previous publication [18]. Group 1 (n = 6) included mice exposed to 0.05% BBN for 12 weeks followed by tap water for 8 weeks (total of 20 weeks). Group 2 (n = 7) included mice treated with 0.05% BBN solution for twelve weeks, maintained with normal tap water for another week, administrated intraperitoneally with mTOR-inhibitor sirolimus (1.5 mg/kg; Wyeth) for five days a week for six consecutively weeks, i.e. until the 19th week, followed by another week of tap water (total of 20 weeks). Group 3 (n = 6) included mice exposed to 0.05% BBN for 12 weeks followed by tap water for 11 weeks (total of 23 weeks). Group 4

Table 2. Antibodies used in the immunohistochemical analysis.

Antibody	Vendor	Clone	Dilution
Tn	Non-commercial Hybridoma*	IE3	1:5
STn	Non-commercial Hybridoma*	TKH2	1:20
Ki-67	Dako	MIB-1	1:100
p53	Dako	DO-7	1:100
Phos-AKT	Cell Signaling	Ser473 (736E11)	1:50
Phos-mTOR	Cell Signaling	Ser2448(49F9)	1:100
Phos-S6	Cell Signaling	Ser240/244 polyclonal	1:75
PTEN	Cell Signaling	D4.3 XP	1:50

*Kindly provided by Prof. Celso Reis (IPATIMUP, UP, Portugal)

doi:10.1371/journal.pone.0141253.t002

(n = 7) included mice treated with 0.05% BBN and sirolimus, as described for Group 2, but with an exposure to tap water afterwards of 3 weeks (total of 23 weeks). Group 3 and 4 were created to estimate the possibility of late relapse and/or molecular alterations resulting from prolonged survival. All procedures were performed in accordance with the European Directive 2010/63/EU. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). The histological changes induced by these experiments included both preneoplastic and neoplastic lesions with invasive potential and invasive tumours, as described in detail by Oliveira et al. [18]. Herein, lesions of high invasive potential and muscle invasive tumours were screened for STn and pS6 by immunohistochemistry, as described in detail for human tumours, since the antibodies used are reactive against both human and mice. Both the intensity and the extension of immunostaining were taken into consideration to score the expression of the antigens, as described in the previous section. The bladder lesions and immunostaining were assessed double-blindly by two independent observers (CC and SP) and validated by an experienced veterinary pathologist (RMGC).

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. Kaplan-Meier survival curves were used to evaluate correlation between STn expression and cancer-specific survival (CSS) and were compared using log-rank test. Furthermore, multivariate Cox regression analysis was performed to assess the individual effect of the evaluated markers on patient's survival and adjust to potential confounders (variables that could affect CSS of NMIBC and MIBC patients). The correlation between PI3K/Akt/mTOR pathway molecules was performed using Spearman rho test.

Results

Altered protein glycosylation, translated by the expression of the STn antigen and its precursor Tn, PI3K/Akt/mTOR pathway molecules (pAkt, pmTOR, pS6), and PTEN inactivation, are salient features of bladder tumours. Herein we have devoted to a comprehensive analysis of these molecular alterations in a series of bladder cancer patients at different stages of the disease, envisaging biomarkers of poor cancer-specific survival.

Our dataset was composed by 47 NMIBC and 49 MIBC patients, as showed in Table 1. According to Fig 2, NMIBC presented a higher cancer-specific survival (CSS; mean CSS: 119 months) than MIBC patients (mean CSS: 43 months; log rank, $p<0.001$). These results

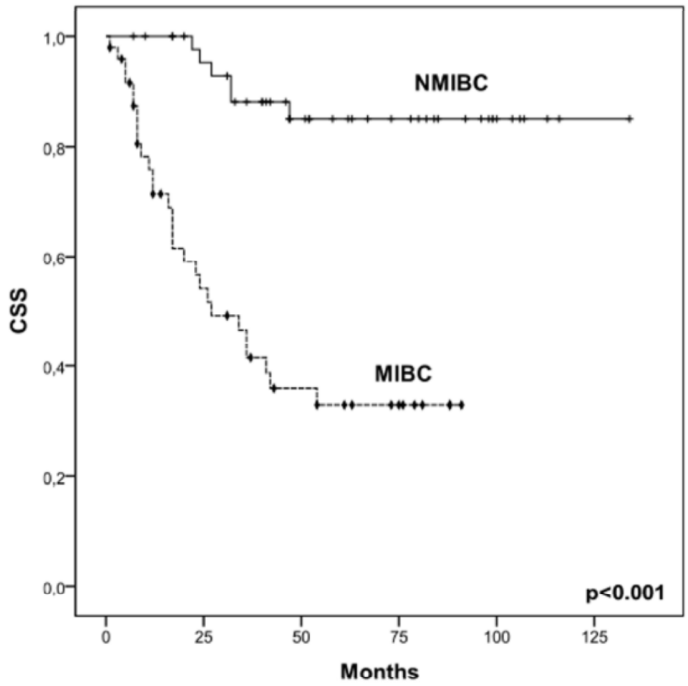

Fig 2. Association between disease groups and cancer-specific survival (CSS) in the studied patients. Kaplan-Meier analysis showing the CSS of NMIBC (Ta and T1) and s of MIBC (T2,T3 and T4). Comparison performed by log-rank test (p<0.001); + censored NMIBC patients; ♦ censored MIBC patients.

doi:10.1371/journal.pone.0141253.g002

demonstrated that our series reflected the natural course of disease and highlighted the significantly lower CSS of MIBC compared to NMIBC cases. Therefore, particular interest was set in the identification of biomarkers for late stage disease based on the comparison between NMIBC and MIBC.

Tn and STn antigen expressions in bladder cancer

The Tn antigen was observed in approximately 10% of NMBC and MIBC (Table 2) and its expression was residual, did not exceeding 5% of the tumour area and without any defined pattern. On the other hand, the STn antigen was detected in approximately 60% of the studied bladder tumours, which is in accordance with our previous findings [5]. The antigen was predominately expressed at the cell membrane, although cytoplasmic staining could also be observed. The STn antigen presented a focal expression that did not exceed 30% of the tumour area for the majority of the positive cases, irrespectively of their histological origin. STn was mainly expressed by dedifferentiated cells in tumours showing *lamina propria* (T1; 60%) and *muscularis propria* (≥ T2; approximately 60–90%) invasion; conversely the percentage of positive Ta was lower than 30% ($p<0.001$; Fig 3A). Although without statistical significance, in Ta tumours STn positive cells were mainly present in superficial tumour layers away from the vessels. Conversely, STn positive cells in T1 tumours (Fig 3B) were observed accompanying and/

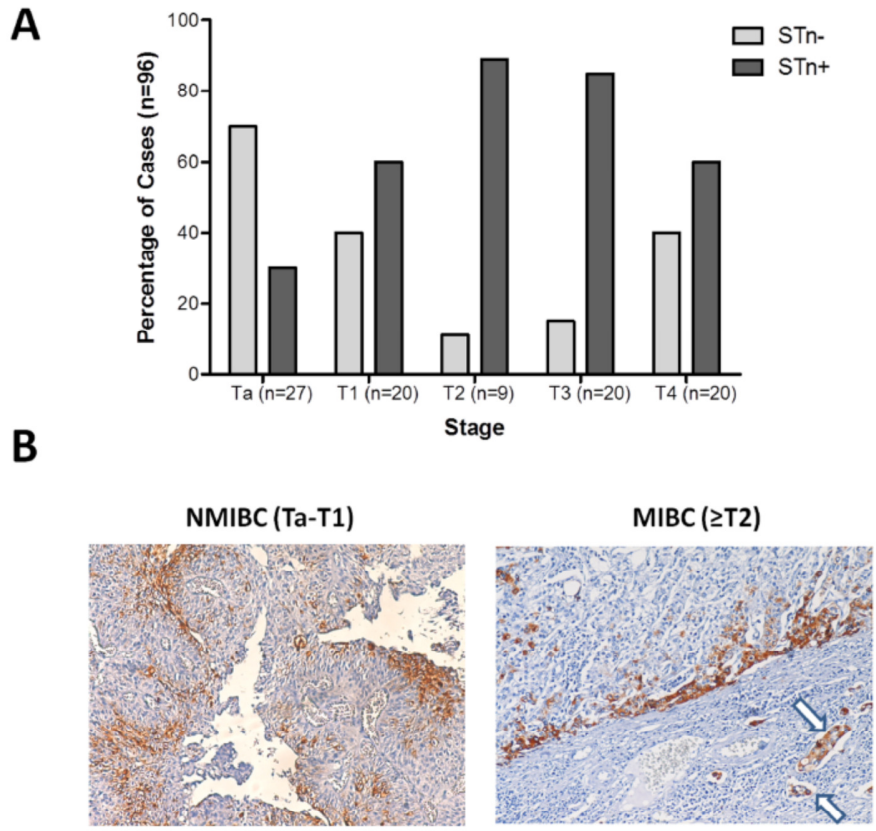

Fig 3. STn expression in different bladder tumors stages. (A) Distribution of STn negative and positive tumors along the different stages of bladder cancer; (B) Representative images of STn staining in NMIBC and MIBC. Left—NMIBC showing a predominance of STn positive cells in the superficial layers, away from the fibrovascular support; note vessels without positive cells. Right—MIBC showing the invasion front with STn positively stained cells; note positive STn urothelial cells in the vessels (arrow), suggesting possible involvement in metastasis.

doi:10.1371/journal.pone.0141253.g003

or invading the basal layer (Fig 3B), while in MIBC these cells were mostly found in the invasion fronts (Fig 3B) and invading and/or inside the vessels, which suggests a role in invasion and disease dissemination. Reinforcing these observations, the presence of STn antigen was statistically higher in MIBC when compared to NMIBC ($p = 0.001$, Table 3).

PI3K/Akt/mTOR pathway in bladder cancer

The evaluation of the PI3K/Akt/mTOR/S6 pathway was done using antibodies for active phosphorylated forms of Akt (pAkt), mTOR (pmTOR), and S6 (pS6). PTEN, that negatively regulates Akt signalling, was also evaluated.

pAkt was detected both in the cytoplasm and nucleus. In NMIBC cases several areas with different intensity of expression were observed (Fig 4A), denoting a heterogeneous pattern that was not evident in MIBC (Fig 4B). Furthermore, stromal cells of MIBC positive cases showed enhanced staining intensity mainly in the areas close to the tumour. pmTOR immunoreactivity

Table 3. Association between the evaluated markers and the stage of disease.

	Bladder Cancer		
	NMIBC n (%)	MIBC n (%)	P
Tn			
Negative	41 (87.2)	45 (91.8)	
Positive	6 (12.8)	4 (8.2)	0.461
STn			
Negative	27 (57.4)	12 (24.5)	
Positive	20 (42.6)	37 (75.5)	0.001
pAKT			
Negative	13 (28.9)	19 (38.8)	
Positive	32 (71.1)	30 (61.2)	0.312
pmTor			
Negative	30 (63.8)	33 (67.3)	
Positive	17 (36.2)	16 (32.7)	0.717
pS6			
Negative	22 (47.8)	28 (57.1)	
Positive	24 (52.2)	21 (42.9)	0.183
PTEN			
Negative	18 (38.3)	37 (82.2)	
Positive	29 (61.7)	8 (17.8)	<0.001

doi:10.1371/journal.pone.0141253.t003

was cytoplasmic and, in occasional cases, nuclear. In urothelium with apparent normal histology pmTOR expression was restricted to superficial cell layers. In NMIBC pmTOR expression was evenly distributed across the several layers of urothelial cells, although there was a more intense staining in the superficial layers (Fig 4C). Moreover, several areas with variable staining intensity were observed, denoting a heterogeneous expression. In MIBC positives cases, pmTOR expression was focal and heterogeneous (Fig 4D). pS6 immunoreactivity was predominantly cytoplasmic. In NMIBC pS6 expression was noted in all the superficial layers, both in umbrella and differentiated cells (Fig 4E). The immunoreactivity of pS6 varied across the tumour cells. In MIBC pS6 presented a diffuse expression throughout the tumour, being more present in basal and mitotic cells (Fig 4F). Several positive cases presented increased pS6 staining intensity in the invasion front as well as pS6 expression in tumour infiltrating lymphocytes and endothelial cells.

Taking into account the extension of staining and its intensity, 62/94 (66%), 33/96 (34%) and 45/95 (47%) of the bladder tumours were considered positive for pAkt, pmTOR and pS6, respectively. A Spearman rho test showed that pAkt, pmTOR, pS6 expressions were significantly correlated ($P<0.05$) irrespectively of the tumour stage, thus in accordance with a fully active pathway. Furthermore, despite histological differences, these markers presented an equal distribution among the NMIBC and MIBC and could not be associated with muscle invasion (Table 3).

On the other hand, 37/92 (40%) of the tumours were considered positive for PTEN. PTEN was expressed in the cytoplasm and nucleus of the same cells, however with lower extension of expression in MIBC (33%, Fig 4G) compared to NMIBC (83%; Fig 4H). Moreover, the PTEN-negative phenotype was significantly associated with muscle invasion (Ta and T1; $p<0.001$, Table 3), which may contribute to maintain an active PI3K/Akt/mTOR/S6 pathway in these cases.

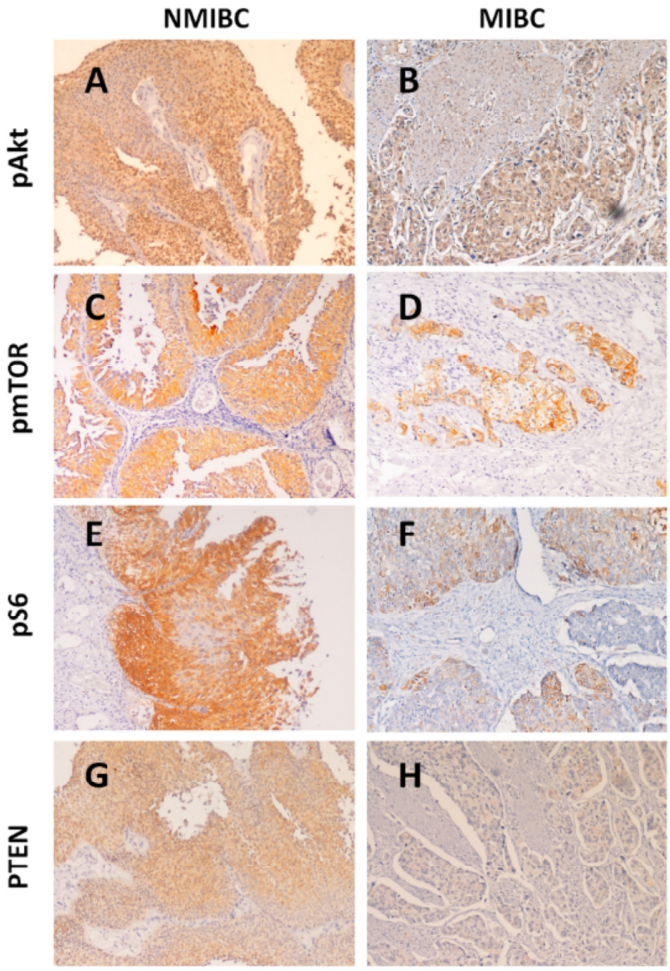

Fig 4. Expressions of pAkt, pmTOR, pS6 and PTEN in NMIBC and MIBC (40x magnification). A and B) pAKT nuclear and cytoplasmatic expression in NMIBC (A) and MIBC (B). In NMIBC cases pAkt presented a heterogeneous pattern with areas of different intensity of expression. In MIBC, stromal cells mainly in the areas close to the tumour showed higher expression. C and D) pmTOR cytoplasmic expression in NMIBC (C) and MIBC (D). In NMIBC pmTOR was expressed across several layers, although there was a more intense staining in the superficial ones. In MIBC positive cases pmTOR expression was focal. E and F) pS6 cytoplasmatic expression in NMIBC (E) and MIBC (F). In NMIBC pS6 expression was observed in all the superficial layers both in umbrella and differentiated cells. In MIBC the immunoreactivity was diffuse, however more present in basal and mitotic cells. pS6 expression was higher in the invasion front and in tumour infiltrating lymphocytes and endothelial cells. G and H) PTEN cytoplasmic and nuclear expressions in NMIBC (G) and MIBC (H). PTEN expression was higher in NMIBC compared to MIBC.

doi:10.1371/journal.pone.0141253.g004

Tn, STn, PI3K/Akt/mTOR pathway and Cancer-specific Survival

A Kaplan-Meier analysis was used to evaluate associations between the addressed biomarkers and the cancer-specific survival of patients. We observed that patients bearing STn expressing tumours had a lower CSS, irrespectively of their stage ($p = 0.024$; Fig 5A). This was also observed when evaluating NMIBC alone ($p = 0.020$; Fig 5B). More importantly, among NMIBC, STn expressing T1 tumours presented lower CSS than negative tumours ($p < 0.05$). Moreover, multivariate Cox regression analysis adjusted to potential confounders, namely age, gender, stage, grade, recurrence status, presence of concomitant CIS was performed. We found that STn is an independent prognostic marker of worst CSS (HR = 11.836; 95%CI: [1.063–131.7]; $p = 0.044$). Contrasting with STn, positive Tn, pAkt, pmTOR and pS6 tumours showed no differences in CSS compared to negative lesions, irrespectively of their stage. We have also observed that patients harbouring PTEN-negative tumours had lower CSS ($p = 0.015$, Fig 6). More studies are necessary to determine if the lack of suppressive effect of PTEN over PI3K/Akt/mTOR may account for these findings.

Based on these observations and aiming to improve the prognostic value of STn in the context of late stage disease (MIBC), we have comprehensively integrated the information from STn and PI3K/Akt/mTOR pathway biomarkers. According to Fig 7, the introduction of PI3K/Akt/mTOR pathway molecules allowed discriminating STn positive MIBC tumours with worst CSS ($p = 0.027$). Furthermore, multivariate Cox regression analysis (adjusted to age, stage, recurrence status, presence of concomitant CIS and metastasis) revealed that the presence of PI3K/Akt/mTOR pathway molecules in STn+ MIBC is independently associated with approximately 6-fold risk of death by cancer (HR = 5.662; 95%CI: [1.093–29.323]; $p = 0.039$). These observations suggest, for the first time, that the combination of STn and mTOR pathway biomarkers may hold potential to improve the stratification of advanced stage bladder tumours; however corroboration in larger series is mandatory.

Inhibition of the PI3K/Akt/mTOR pathway in animal models

BBN-induced mice bladder tumours mimicking the histology and molecular nature of human cancers [20,21], were screened for STn and pS6, the downstream effector of mTOR pathway. We observed no STn expression in the healthy mice urothelium, in accordance with previous observation for the healthy human bladder [5]. In mice healthy urothelium pS6 expression was below 20%, thus underexpressed when compared with BBN-exposed mice (Fig 8). In the control groups (Group 1 and 3, Fig 8A), the exposure to BBN led to the development of invasive tumours in 70–90% of the studied mice. Concomitantly, 83–100% of the invasive lesions overexpressed the STn antigen and all significantly overexpressed pS6 (Fig 8B). This demonstrated that BBN-induced lesions were able to recapitulate the association between altered glycosylation and an activated PI3K/Akt/mTOR pathway previously observed in advanced stage human tumours. The STn antigen was mainly found in cells adjacent to the basal layer and in those invading the stroma, as previously observed in human tumours (Fig 8B and 8C). Conversely, pS6 presented a more diffuse expression, again in accordance with the pattern observed in human lesions (Fig 8B and 8C). A comparison between groups 1 and 3 further highlighted that extended lifespan did not alter the number of invasive lesions, but significantly increased STn and pS6 overall expressions in each tumour ($p < 0.05$; Fig 8B), highlighting the more aggressive nature of Group 3 lesions.

In the sirolimus-treated groups (Groups 2 and 4; Fig 8A) a smaller number of mice developed invasive tumors (20–40%) when compared to the controls (Groups 1 and 3). Moreover, only 43% of the mice treated with sirolimus overexpressed the STn antigen, irrespectively of the experience periods. Still, the extension of STn expression was significantly decreased in

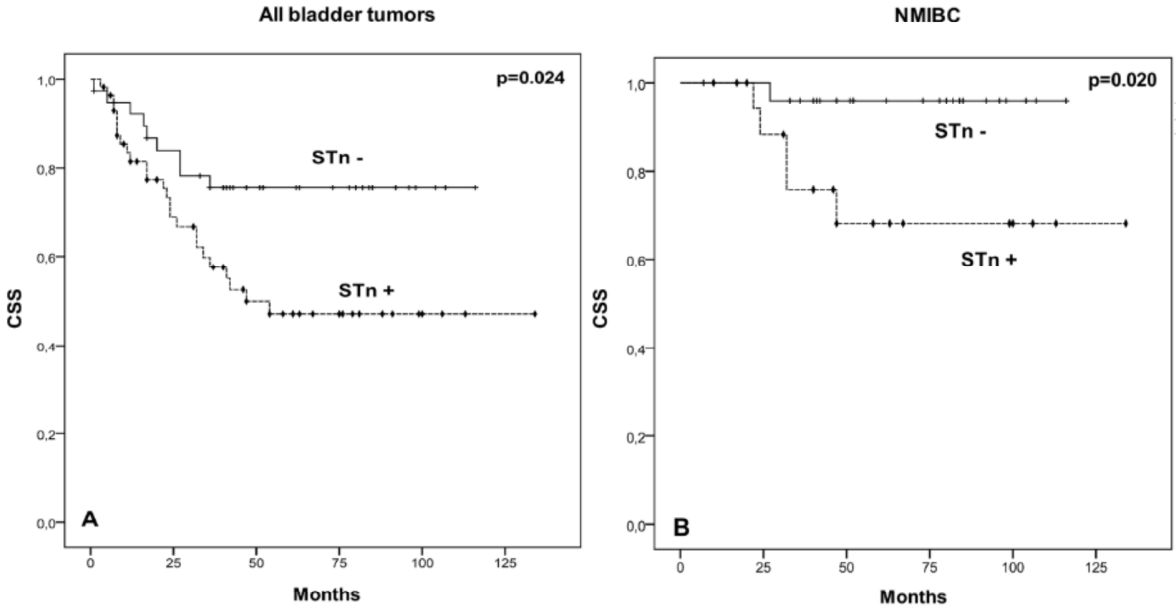

Fig 5. Effect of STn expression in cancer-specific survival (CSS). Kaplan–Meier analysis showing the association between STn and CSS in: (A) all studied bladder cancer patients; (B) NMIBC patients. Comparison performed by log-rank test (A: p = 0.024; B: p = 0.020); + censored STn negative tumours; ♦ censored STn positive tumours.

doi:10.1371/journal.pone.0141253.g005

STn-positive tumours when compared to the control groups (Fig 8B and 8C). Following the same tendency, the pS6 protein was only overexpressed in 29% of the cases in Group 2 and the extension of expression was also significantly decreased (Fig 8B and 8C). Contrastingly, the expression of pS6 in Group 4 was higher than in Group 2, again translating the higher aggressive nature of tumours obtained after longer lifespan. Despite these observations, sirolimus treatment promoted a significant reduction in the percentage of positive pS6 cells in Group 4 mice when compared to Group 3 ($p<0.05$; Fig 8B and 8C). Altogether, sirolimus administration effectively reduced tumour burden and promoted a significant reduction in the expression of STn and pS6 markers.

Discussion

Due to their high molecular heterogeneity, advanced stage bladder tumours present a significant prognostication and treatment hurdle. In this context, much controversy exists regarding the potential of conventional cancer biomarkers, urging the identification of novel molecules capable of aiding disease personalization. Furthermore, advanced stage bladder cancer remains an orphan disease in terms of therapeutics, as the only available options continue to be surgery and conventional chemotherapy [22]. The introduction of targeted therapeutics is therefore warranted.

In a previous explorative study we have observed that altered protein glycosylation translated by STn overexpression was a salient feature of a subset of advanced stage tumours [5]. Herein we have started by investigating the expression of STn precursor, the Tn antigen, in bladder tumours. We observed that this antigen presented a very low expression in bladder

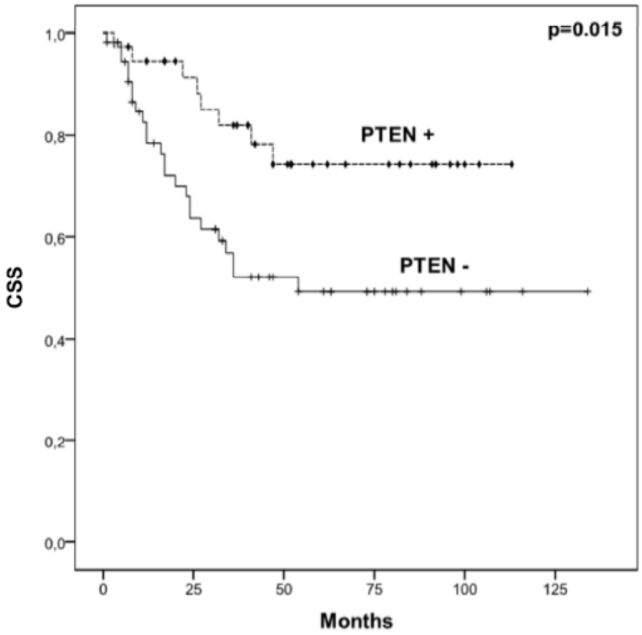

Fig 6. Effect of PTEN expression and cancer-specific survival (CSS) in the studied patients. Kaplan-Meier analysis showing the effect in CSS of PTEN expression in all studied bladder cancer patients. Comparison performed by log-rank test (p = 0.013); + censored PTEN negative tumours; ♦ censored PTEN positive tumours.

doi:10.1371/journal.pone.0141253.g006

tumours and was not associated with any particular stage of the disease. These findings suggest that the Tn antigen is rapidly sialylated or capped with more extended glycans in bladder tumours. Moreover, we have confirmed that STn expression is more associated with muscle invasive than non-muscle invasive disease in a larger patient set, suggesting that sialylation plays a key role in stopping protein glycosylation in advanced stage bladder tumours. Furthermore, we have provided new insights regarding its correlation with decreased survival, as previously observed for digestive track tumours [23–25]. Accordingly, we and other authors have shown that STn expression is responsible by the modulation of cell surface glycoprotein functions in ways that favour malignant phenotypes in gastric [26], breast [27] and bladder [5] cancers. Namely, STn expression altered the adhesive properties of cancer cells, possibly by impairing integrin function [26,27]. Furthermore, it enhanced cell motility, invasion [26,27] and epithelial-to-mesenchymal transition, a key event leading to metastasis [28]. We have also demonstrated that STn expression protects bladder cancer cells from adverse host immune responses [6]. Namely, it impaired dendritic cell maturation inducing a tolerogenic phenotype and limiting their capacity to trigger protective anti-tumour T-cell responses [6]. In resume, a significant amount of data supports a key role of STn in disease progression and dissemination, making of STn antigen, and in particular STn-glycoproteins, potential anti-cancer targets. Nevertheless, there is scarce information about the molecular nature of this subset of STn-expressing aggressive tumours and consequently about the best therapeutic options.

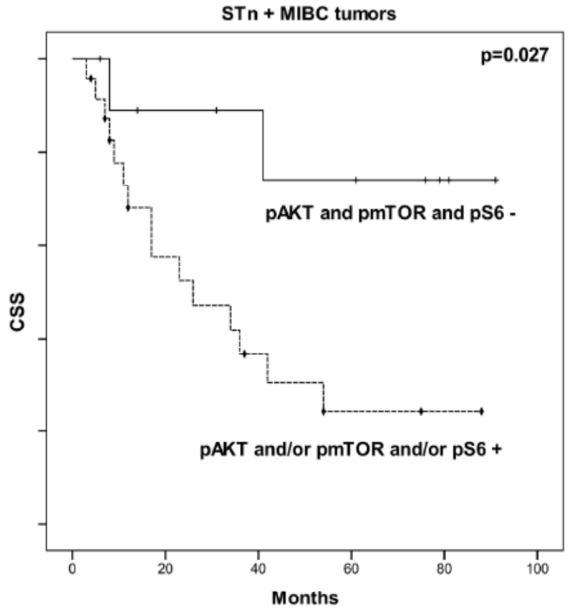

Fig 7. Effect of PI3K/Akt/mTOR pathway activation in cancer-specific survival (CSS) of patients with STn positive MIBC. Kaplan–Meier analysis showing the association between pAKT, pmTOR and pS6 expressions in the CSS of STn positive tumors MIBC: Comparison performed by log-rank test (p = 0.027); + censored pAKT and pmTOR and pS6 negative tumours; ♦ censored pAKT and/or pmTOR and/or pS6 positive tumours.

doi:10.1371/journal.pone.0141253.g007

Foreseeing a more accurate patient stratification we have also addressed the expression of PI3K/Akt/mTOR pathway markers in bladder tumours. In our series the activation of mTOR pathway proteins did not discriminate the stage of disease. Moreover it did not allow, by itself, the identification of patients facing worst prognosis, which is in accordance with recent publications [29,30]. However, we found that PTEN expression, which exerts a suppressive effect over the PI3K/Akt/mTOR pathway, was decreased in advanced stage tumours, in accordance with previous observations [31–34]. Furthermore, PTEN-negative MIBC presented worst cancer-specific survival in comparison to PTEN-positive lesions. More studies are needed to determine if the lack of suppressive effect over the PI3K/Akt/mTOR may account for poorer outcome. Interestingly, we have also observed that the overexpression of PI3K/Akt/mTOR pathway biomarkers decisively associated with worst CSS in STn positive advanced stage tumours, which currently lack effective therapeutics. These findings lead us to hypothesize that this subset of more aggressive bladder tumours may benefit from multi-targeted approaches combining mTOR-inhibitors and guided therapeutics against STn-expressing cells. However these are preliminary insights from a relatively low number of patients. More studies involving a large population are warranted to confirm these observations. It will also be important to evaluate other outcomes of aggressiveness, namely response to conventional therapeutics and metastasis development.

Our study also reinforced that bladder tumours present extensive activation of the PI3K/Akt/mTOR pathway irrespectively of their histological nature, as described in previous publications [32,35]. Such findings contribute to support the idea that most bladder tumours may be good candidates for mTOR-inhibitors therapeutics. Accordingly, mTOR-inhibitors have been

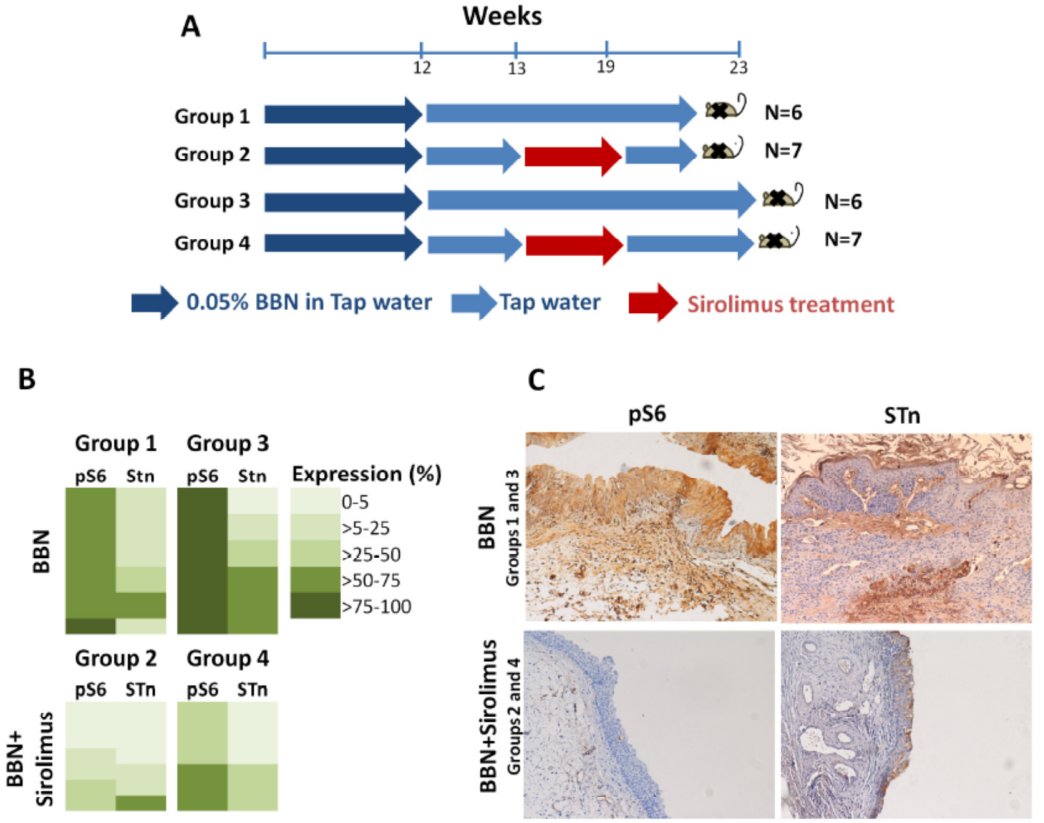

Fig 8. STn and pS6 expressions in bladder tumours from BBN-exposed male ICR mice with or without the administration of mTOR-inhibitor sirolimus (rapamycin). A) Experimental design to determine the sirolimus effect on STn and pS6 expressions in a model of urothelial carcinogenesis (male ICR mice). B) Expression of STn and pS6 in BBN-derived urothelial tumours in the presence and absence of sirolimus. BBN-induced bladder tumours (Groups 1 and 3) overexpressed STn and pS6, which was more pronounced in Group 3, after longer lifespan. Exposure to sirolimus decreased the number of invasive lesions in groups 2 and 4 (data not shown) and, concomitantly, decreased the expressions of STn and pS6. C) Histological sections showing the expressions of STn and pS6 in BBN-induced urothelial tumours before and after treatment.

doi:10.1371/journal.pone.0141253.g008

extensively explored in pre-clinical settings and two phase I/II clinical trials for bladder cancer are ongoing [36]. In particular our group has demonstrated that the combination of everolimus with cisplatin or gemcitabine decreased the proliferation of bladder cancer cell lines in comparison to the chemotherapy agent alone [14,37]. More recently we conducted studies in mice bearing chemically-induced tumours mimicking the histological and molecular nature of human tumours [20]. We concluded that administration of mTOR-pathway inhibitor sirolimus (rapamycin) effectively reduced the frequency of invasive lesions. Using the same animal model, we have now confirmed the anti-cancer activity of sirolimus in the context of aggressive bladder disease. Namely, we observed a significant reduction in tumour burden accompanied by a loss of pS6 expression, thus in accordance with the expected mechanism of action of the drug. Moreover, we are describing for the first time that chemically-induced bladder tumours expressed the

STn antigen, thereby mimicking the glycosylation pattern of human cancers. These observations are of particular importance due the lack of accurate models to access the biological role of this antigen. In fact most established cancer cell lines express residual amounts of this antigen, denoting a dependence on the tumours microenvironment. We believe that BBN-induced tumours may now constitute key models to develop successful therapeutics against STn positive bladder lesions. Moreover importantly, we have concluded that the administration of sirolimus contributed to reduce the number of STn positive cells. These observations reinforce a possible association between STn and an active PI3K/Akt/mTOR pathway in invasive tumours, as suggested upon the evaluation of human cancers. It also points out that sirolimus may constitute a valuable approach to manage STn and PI3K/Akt/mTOR-positive, which face worst OS. Still, these preliminary evidences and more in depth studies are needed before progressing to clinical phases. Namely, it will be important to support these findings in other models such as patient-derived xenografts and compare the effect of sirolimus with conventional chemotherapeutics for bladder cancer (cisplatin/gemcitabine-based regimens).

In resume, we have demonstrated that the STn antigen is a biomarker of poor prognosis, particularly in MIBC. We also suggest the existence of potentially more aggressive subgroup of STn positive MIBC characterized by an active mTOR-pathway. Such observations also provide the first link between these two apparently unrelated events in bladder cancer (altered glycosylation and the PI3K/Akt/mTOR-pathway activation). Using animal models we have also concluded that the administration of mTOR-pathway inhibitor sirolimus offers potential against these highly malignant tumours. More validation studies are now warranted to set the pace for clinical trials. Taking into consideration its cell-surface nature and key role played by STn malignancy, specific antibody-based therapeutics can also be envisaged [22,38]. The combination of these approaches may provide novel ways to improve MIBC management, which remains an orphan disease in terms of innovative treatments [22].

Acknowledgments

This work was supported by Portuguese Foundation for Science and Technology (FCT) Post-doctoral grants SFRH/BPD/66288/2009 (José Alexandre Ferreira), SFRH/BPD/101827/2014 (Luis Lima), SFRH/BPD/85462/2012 (Rui Gil da Costa) and PhD grants SFRH/BD/103571/2014 (Elisabete Fernandes) and SFRH/BD/111242/2015 (Andreia Peixoto). FCT is co-financed by European Social Fund (ESF) under Human Potential Operation Programme (POPH) from National Strategic Reference Framework (NSRF). The authors also acknowledge financial support from ICBAS-UP (Céu Costa and Sofia Pereira) and the Portuguese Association of Urology/Pfizer prize 2013. The authors also thank Professor Celso Reis (IPATIMUP, UP, Portugal) by kindly providing the anti-STn TKH2 and the anti-Tn IE3 monoclonal antibodies used in this work.

Author Contributions

Conceived and designed the experiments: CC SP LL PO JAF LLS. Performed the experiments: CC SP AP EF AT DN MN CG RMGC PO. Analyzed the data: CC SP LL RMGC RC TA PO JAF LLS. Contributed reagents/materials/analysis tools: RC PO JAF LLS. Wrote the paper: LL JAF LLS PO.

References

1. Pasin E, Josephson DY, Mitra AP, Cote RJ, Stein JP. Superficial bladder cancer: an update on etiology, molecular development, classification, and natural history. Rev Urol. 2008; 10: 31–43. PMID: 18470273

2. Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BWG, Compérat E, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. Eur Urol. 2013; 64: 639–53. doi: 10.1016/j.eururo.2013.06.003 PMID: 23827737
3. Miakhil I, Parker SG, Kommu SS, Nethercliffe J. A review of molecular biomarkers for bladder cancer. Int J Med Biomed Res. 2013; 2: 186–194.
4. Dovedi SJ, Davies BR. Emerging targeted therapies for bladder cancer: a disease waiting for a drug. Cancer Metastasis Rev. 2009; 28: 355–67. doi: 10.1007/s10555-009-9192-9 PMID: 19997963
5. Ferreira JA, Videira PA, Lima L, Pereira S, Silva M, Carrascal M, et al. Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol. 2013; 7: 719–31. doi: 10.1016/j.molonc.2013.03.001 PMID: 23567325
6. Carrascal MA, Severino PF, Guadalupe Cabral M, Silva M, Ferreira JA, Calais F, et al.Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. Mol Oncol. 2014; 8: 753–65. doi: 10.1016/j.molonc.2014.02.008 PMID: 24656965
7. Dall'Olio F, Malagolini N, Trinchera M, Chiricolo M. Mechanisms of cancer-associated glycosylation changes. Front Biosci (Landmark Ed). 2012; 17: 670–99.
8. Ma H, Zhou H, Song X, Shi S, Zhang J, Jia L. Modification of sialylation is associated with multidrug resistance in human acute myeloid leukemia. Oncogene. 2015; 34: 726–40. doi: 10.1038/onc.2014.7 PMID: 24531716
9. Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR Signaling in Cancer. Front Oncol. 2014; 4: 1–11.
10. Ching CB, Hansel DE. Expanding therapeutic targets in bladder cancer: the PI3K/Akt/mTOR pathway. Lab Invest. Nature Publishing Group; 2010; 90: 1406–1414.
11. Wu X, Obata T, Khan Q, Highshaw RA, De Vere White R, Sweeney C. The phosphatidylinositol-3 kinase pathway regulates bladder cancer cell invasion. BJU Int. 2004; 93: 143–150. PMID: 14678387
12. Keniry M, Parsons R. The role of PTEN signaling perturbations in cancer and in targeted therapy. Oncogene. 2008; 27: 5477–5485. doi: 10.1038/onc.2008.248 PMID: 18794882
13. Tamura M, Gu J, Tran H, Yamada KM. PTEN gene and integrin signaling in cancer. J Natl Cancer Inst. 1999; 91: 1820–1828. PMID: 10547389
14. Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, Gaivão I, Cardoso ML, Colaço A, et al. Everolimus enhances gemcitabine-induced cytotoxicity in bladder-cancer cell lines. J Toxicol Env Heal A. 2012; 75: 788–99.
15. Munari E, Fujita K, Faraj S, Chaux A, Gonzalez-Roibon N, Hicks J, et al. Dysregulation of mammalian target of rapamycin pathway in upper tract urothelial carcinoma. Hum Pathol. Elsevier Inc.; 2013; 44: 2668–2676.
16. Nishikawa M, Miyake H, Behnsawy HM, Fujisawa M. Significance of 4E-binding protein 1 as a therapeutic target for invasive urothelial carcinoma of the bladder. Urol Oncol. 2015; 33: 166.e9–15.
17. Chaux A, Compérat E, Varinot J, Hicks J, Lecksell K, Solus J, et al. High levels of phosphatase and tensin homolog expression are associated with tumor progression, tumor recurrence, and systemic metastases in pt1 urothelial carcinoma of the bladder: A tissue microarray study of 156 patients treated by transurethral resect. Urology. 2013; 81: 116–122. doi: 10.1016/j.urology.2012.09.007 PMID: 23273076
18. Oliveira PA, Arantes-Rodrigues R, Sousa-Diniz C, Colaço A, Lourenço L, De La Cruz P LF, et al. The effects of sirolimus on urothelial lesions chemically induced in ICR mice by BBN. Anticancer Res. 2009; 29: 3221–3226. PMID: 19661338
19. Lima L, Severino PF, Silva M, Miranda A, Tavares A, Pereira S et al. Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy. Br J Cancer. 2013; 109: 2106–14. doi: 10.1038/bjc.2013.571 PMID: 24064971
20. Vasconcelos-Nóbrega C, Colaço A, Lopes C, Oliveira PA. BBN as an urothelial carcinogen. In Vivo (Brooklyn). 2012; 26: 727–739.
21. Palmeira C, Oliveira PA, Lameiras C, Amaro T, Silva VM, Lopes C, et al. Biological similarities between murine chemical-induced and natural human bladder carcinogenesis. Oncol Lett. 2010; 1: 373–377. PMID: 22966311
22. Azevedo R, Ferreira JA, Peixoto A, Neves M, Sousa N, Lima A et al. Emerging antibody-based therapeutic strategies for bladder cancer: A systematic review. J Control Release. 2015;
23. Flucke U, Zirbes T, Schröder W, Mönig S, Koch V, Schmitz K, et al. Expression of mucin-associated carbohydrate core antigens in esophageal squamous cell carcinomas. Anticancer Res. 2001; 21: 2189–2193. PMID: 11501845
24. Victorzon M, Nordling S, Nilsson O, Roberts P, Haglund C. Sialyl Tn antigen is an independent predictor of outcome in patients with gastric cancer. Int J Cancer. 1996; 65: 295–300. PMID: 8575847

25. Tsuchiya A, Kikuchi Y, Ando Y, Abe R. Correlation between expression of sialosyl-T antigen and survival in patients with gastric cancer. Br J Surg. 1995; 82: 960–962. PMID: 7648120

26. Pinho S, Marcos NT, Ferreira B, Carvalho AS, Oliveira MJ, Santos-Silva F, et al. Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett. 2007; 249: 157–70. PMID: 16965854

27. Julien S, Adriaenssens E, Ottenberg K, Furlan A, Courtand G, Vercoutter-Edouart AS, et al. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology. 2006; 16: 54–64. PMID: 16135558

28. Lin J-C, Liao S-K, Lee E-H, Hung M-S, Sayion Y, Chen H-C, et al. Molecular events associated with epithelial to mesenchymal transition of nasopharyngeal carcinoma cells in the absence of Epstein-Barr virus genome. J Biomed Sci. 2009; 16: 105. doi: 10.1186/1423-0127-16-105 PMID: 19930697

29. Korkolopoulou P, Levidou G, Trigka EA, Prekete N, Karlou M, Thymara I, et al. A comprehensive immunohistochemical and molecular approach to the PI3K/AKT/mTOR (phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene/mammalian target of rapamycin) pathway in bladder urothelial carcinoma. BJU Int. 2012; 110: 1237–1248.

30. Fahmy M, Mansure JJ, Brimo F, Yafi FA, Segal R, Althunayan A, et al. Relevance of the mammalian target of rapamycin pathway in the prognosis of patients with high-risk non–muscle invasive bladder cancer. Hum Pathol. Elsevier Inc.; 2013; 44: 1766–1772.

31. Sun CH, Chang YH, Pan CC. Activation of the PI3K/Akt/mTOR pathway correlates with tumour progression and reduced survival in patients with urothelial carcinoma of the urinary bladder. Histopathology. 2011; 58: 1054–1063. doi: 10.1111/j.1365-2559.2011.03856.x PMID: 21707707

32. Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She Q-B, Maurer M, et al. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci U S A. 2007; 104: 7564–7569. PMID: 17452630

33. Harris L, De La Cerda J, Tuziak T, Rosen D, Xiao L, Shen Y, et al. Analysis of the Expression of Biomarkers in Urinary Bladder Cancer Using a Tissue Microarray. Mol Carcinog. 2008; 47: 678–685. doi: 10.1002/mc.20420 PMID: 18288642

34. Calderaro J, Rebouissou S, de Koning L, Masmoudi A, Hérault A, Dubois T, et al. PI3K/AKT pathway activation in bladder carcinogenesis. Int J cancer. 2014; 134: 1776–84. doi: 10.1002/ijc.28518 PMID: 24122582

35. Ferreira J, Bernardo C, Amaro T, Costa C, Lopes P, Silva V, et al. Patient-derived sialyl-Tn positive invasive bladder cancer xenografts in nude mice: An exploratory model study. Eur Urol Suppl. European Association of Urology; 2014; 13: e518.

36. Massari F, Ciccarese C, Santoni M, Brunelli M, Conti A, Modena A, Montironi R, Santini D, Cheng L, Martignoni G, Cascinu S TG. The route to personalized medicine in bladder cancer: where do we stand? Target Oncol. 2015;

37. Kjeldsen T, Clausen H, Hirohashi S, Ogawa T, Iijima H, Hakomori S. Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2——6 alpha-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res. 1988; 48: 2214–2220. PMID: 2450649

38. Fernandes E and Ferreira JA, Andreia P, Luís L, Barroso S, Sarmento B, et al. New trends in guided nanotherapies for digestive cancers: A systematic review. J Control Release. 2015; 209: 288–307. doi: 10.1016/j.jconrel.2015.05.003 PMID: 25957905

3.1.1 Paper II

Bladder cancer-induced skeletal muscle wasting: disclosing the role of mitochondria plasticity.

Padrão AI, Oliveira P, Vitorino R, Colaço B, Pires MJ, Márquez M, Castellanos E, Neuparth MJ, Teixeira C, **Costa C**, Moreira-Gonçalves D, Cabral S, Duarte JA, Santos LL, Amado F, Ferreira R.

Int J Biochem Cell Biol. 2013. Jul; 45(7):1399-409. doi: 10.1016/j.biocel.2013.04.014.

The International Journal of Biochemistry & Cell Biology 45 (2013) 1399–1409

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

Journal homepage: www.elsevier.com/locate/biocel

Bladder cancer-induced skeletal muscle wasting: Disclosing the role of mitochondria plasticity

Ana Isabel Padrão[a], Paula Oliveira[b], Rui Vitorino[a], Bruno Colaço[b], Maria João Pires[b], Marcela Márquez[c], Enrique Castellanos[c], Maria João Neuparth[d], Catarina Teixeira[a,b], Céu Costa[e], Daniel Moreira-Gonçalves[f], Sónia Cabral[g], José Alberto Duarte[f], Lúcio Lara Santos[e,g], Francisco Amado[a,h], Rita Ferreira[a,*]

[a] QOPNA, Chemistry Department, University of Aveiro, Aveiro, Portugal
[b] CECAV, Department of Veterinary Sciences, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
[c] Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden
[d] CITS, IPSN, CESPU CRL, Famalicão, Portugal
[e] Health Faculty, Fernando Pessoa University, Porto, Portugal
[f] CIAFEL, Faculty of Sport, University of Porto, Porto, Portugal
[g] Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Porto, Portugal
[h] Health School of Sciences, University of Aveiro, Aveiro, Portugal

ARTICLE INFO

Article history:
Received 18 October 2012
Received in revised form 15 March 2013
Accepted 14 April 2013
Available online 19 April 2013

Keywords:
Urothelial carcinoma
Gastrocnemius wasting
Mitochondrial proteolysis
Protein quality control system

ABSTRACT

Loss of skeletal muscle is a serious consequence of cancer as it leads to weakness and increased risk of death. To better understand the interplay between urothelial carcinoma and skeletal muscle wasting, cancer-induced catabolic profile and its relationship with muscle mitochondria dynamics were evaluated using a rat model of chemically induced urothelial carcinogenesis by the administration of N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN). The histologic signs of non-muscle-invasive bladder tumors observed in BBN animals were related to 17% loss of body weight and high serum levels of IL-1β, TNF-α, TWEAK, C-reactive protein, myostatin and lactate and high urinary MMPs activities, suggesting a catabolic phenotype underlying urothelial carcinoma. The 12% loss of *gastrocnemius* mass was related to mitochondrial dysfunction, manifested by decreased activity of respiratory chain complexes due to, at least partially, the impairment of protein quality control (PQC) systems involving the mitochondrial proteases paraplegin and Lon. This was paralleled by the accumulation of oxidatively modified mitochondrial proteins. In overall, our data emphasize the relevance of studying the regulation of PQC systems in cancer cachexia aiming to identify therapeutic targets to counteract muscle wasting.

1. Introduction

The majority of patients with advanced cancer experience involuntary weight loss (Blum et al., 2011; Tisdale, 2009), which by itself is responsible for 25–30% of cancer-related deaths (Bonetto et al., 2011). Patients with pancreatic or gastric cancer have the highest frequency of weight loss (Tisdale, 2009) while urological cancer is not usually associated with significant cachexia (Buskermolen et al., 2012). Nevertheless, in a population of around 200 patients with bladder cancer we verified (data not published) that 48% were at risk of undernutrition and 23% showed clear signs of cancer-related body wasting, according to the Buzby's Prognostic Nutritional Index (Dempsey et al., 1983). Though over the last two decades trends in urologic cancer mortality have been favourable, it stills the 9th leading cause of death from cancer in men (Mistry et al., 2011; Jacobs et al., 2012).

The presence of muscle wasting is usually associated with intolerance to treatment, poor quality of life and high mortality in patients (Wang et al., 2012). So, a better understanding of the molecular processes underlying cancer-induced skeletal muscle wasting is critical in planning therapeutic strategies focused on both quality and longevity of patients. In this context, well characterized animal models that mimic human clinical conditions are crucial. Nitrosamine-induced tumorigenesis in rat bladder is a valuable animal model for the study of urothelial carcinoma and related complications (Palmeira et al., 2010).

Among the molecular mechanisms underlying cancer-induced muscle wasting, it has been suggested that tumor and host-derived cytokines affect normal homeostasis and energy metabolism,

* Corresponding author at: Chemistry Department, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal. Tel.: +351 234370700; fax: +351 234370084.
E-mail address: ritaferreira@ua.pt (R. Ferreira).

1357-2725/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2013.04.014

resulting in altered muscle respiration, muscle fatigue and degradation (Shum et al., 2012). A complex network of cytokine signaling encompassing raised levels of tumor-derived IL-1β, IL-6, TNF-alpha has been implicated in the pathogenesis of cancer cachexia (Shum et al., 2012). TGF-β ligands like myostatin, which function through ActRIIB-mediated signaling are also associated with the development of skeletal muscle wasting (Lokireddy et al., 2012; Zhou et al., 2010), which was related to the disruption of the delicate balance between the rates of muscle protein synthesis and degradation (White et al., 2011). The involvement of mitochondrial dysfunction in skeletal muscle wasting was recently reported (Wang et al., 2012; White et al., 2011, 2012; Julienne et al., 2012). The activation of TNF-alpha-induced NFκB was shown to decrease the expression of regulatory factors involved in mitochondrial biogenesis and affect downstream oxidative proteins and consequently disturb muscle oxidative capacity (Wang et al., 2012; Julienne et al., 2012). Morphological changes such as the presence of electron-lucent areas and swelling were also reported in muscle mitochondria of C26-bearing mice (Shum et al., 2012). However, the underlying mechanisms are not known.

Using a rat model of N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced urothelial carcinogenesis, we investigated the interplay between urothelial carcinoma-induced catabolic profile and skeletal muscle phenotype focusing on muscle mitochondrial plasticity. Data obtained highlight an association between mitochondrial dysfunction and the accumulation of oxidized proteins in *gastrocnemius* muscle from BBN animals due to the impairment of protein quality control (PQC) systems.

2. Materials and methods

2.1. Chemicals

N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) was purchased from Tokyo Kasey Kogyo (Japan). All other reagents and chemicals used were of highest grade of purity commercially available. Rabbit polyclonal anti-paraplegin antibody (sc-135026) was obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Rabbit polyclonal anti-mtTFA antibody (ab47548), mouse monoclonal anti-ATPB antibody (ab14730), rabbit monoclonal anti-CRP antibody (C reactive protein; ab32412), rabbit polyclonal anti-GDF8 antibody (myostatin; ab996), rabbit polyclonal anti-LONP1 antibody (ab103809), rabbit monoclonal anti-TRAF6 (ab33915), rabbit polyclonal anti-TWEAK (ab37170), rabbit polyclonal anti-MURF1 (ab77577), rabbit polyclonal anti-phospho Smad3 (S423 and S425; ab51451) and rabbit polyclonal anti-IL-6 (ab6672) were acquired from Abcam (Cambridge, UK). Rabbit polyclonal anti-DNP antibody was obtained from DakoCytomation (Hamburg; Germany). Mouse monoclonal anti-3-nitrotyrosine antibody (#06-284; Millipore) was obtained from Chemicon (CA, USA). Mouse monoclonal MMP-9 (Clone 36020; MAB936) was acquired from R&D systems (Minneapolis, USA). Rabbit polyclonal atrogin-1 antibody (AP2041) was obtained from ECM BioAciences (KY, USA). Rabbit polyclonal antibodies for p-mTOR (#2971) and p-Akt (#4058), and rabbit monoclonal p-4E-BP1 (#2855) and p-p70S6K (#9234) were acquired from Cell Signalling Technology (MA, USA). Secondary peroxidase-conjugated antibodies (anti-mouse IgG and anti-rabbit IgG) were obtained from GE Healthcare (UK). ELISA kit for the determination of IL-1β was acquired from R&D (cat. no RLB00). Colorimetric kit from RANDOX (LC2389; UK) was used to measure serum lactate.

2.2. Animals

Twenty three female Wistar rats were obtained at the age of 5 weeks from Harlan (Barcelona, Spain). During the experimental protocol, animals were housed in groups of 4 rats/cage, in a controlled environment at 22 ± 3 °C and 60 ± 10% of relative humidity with 12:12 h light-dark cycle, with free access to food and water. After a week of acclimatization, the animals were randomly divided into two groups: control (CONT; $n = 10$) and urothelial carcinogenesis (BBN; $n = 13$). The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation.

2.3. Induction and characterization of BBN-induced urothelial carcinogenesis

In order to induce urothelial carcinogenesis, one group of animals was treated with N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN group), which was administered in the drinking water, in light impermeable bottles, at a concentration of 0.05%. No chemical supplementation was added to the drinking water of control animals. The urothelial carcinogenesis group was exposed to BBN for 20 weeks and was maintained with normal tap water until the end of the experiment. After 28 weeks, all animals were sacrificed with 0.4% sodium pentobarbital (1 mL/kg, intraperitoneal) and blood was collected from heart. The urinary bladders were inflated in situ by injection of 10% phosphate-buffered formalin (300 μL), ligated around the neck to maintain proper distension and then were immersed in the same solution for 12 h. After fixation, the formalin was removed; the urinary bladder was cut into strips and was routinely processed for haematoxylin and eosin staining. Urine was collected one week before the end of the experimental protocol with the use of metabolic cages (after a 48 h adaptation period).

2.4. Histopathological analysis

All sections were reviewed by two researchers and the urothelial lesions staged by the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (Epstein et al., 1998). The urothelial lesions were categorized as simple hyperplasia, nodular hyperplasia, papillary hyperplasia, dysplasia, carcinoma *in situ* (CIS), papilloma, papillary neoplasm of low malignant potential, low-level papillary carcinoma, high-level papillary carcinoma, invasive carcinoma and epidermoid metaplasia.

2.5. Blood tests

Serum albumin, total protein, cholesterol, HDL-cholesterol and triglycerides were measured in duplicate on an AutoAnalyzer (PRESTIGE 24i, Cormay PZ). IL-1β serum levels were detected by an ELISA kit used according to the manufacturer instructions (R&D). Serum from each animal was assayed in triplicate. Serum lactate was measured with a commercial kit (RANDOX). Serum C-reactive protein, IL-6, TNF-a, TWEAK and myostatin levels were assayed by immunoblotting as described below.

2.6. Mitochondria isolation from gastrocnemius muscle

The *gastrocnemius* muscles were extracted during animals' sacrifice for the preparation of isolated mitochondria, as previously described (Tonkonogi and Sahlin, 1997). All the procedures were performed on ice or below 4 °C. Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing 100 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid, 50 mM Tris–HCl, 100 mM KCl, 1 mM KH_2PO_4, and 0.2% free fatty acid bovine serum albumin (BSA), pH 7.4. Minced blood-free tissue was rinsed and suspended in 10 mL of fresh medium containing 0.2 mg/mL bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma, MO) and stirred for 2 min. The sample was then carefully homogenized

with a tightly fitted Potter–Elvehjem homogenizer and a Teflon pestle. An aliquot of whole homogenate was reserved for biochemical analysis. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then centrifuged at 700 *g* for 10 min. The resulting supernatant suspension was centrifuged at 10,000 *g* for 10 min. The pellet was gently resuspended in the isolation medium (1.3 mL/100 mg initial tissue) and centrifuged at 7000 *g* for 3 min. The final pellet, containing the mitochondrial fraction, was gently resuspended (0.4 mL/mg initial tissue) in a medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris, and 0.1 mM EDTA, pH 7.4.

Mitochondrial fraction was aliquoted for subsequent biochemical analysis. Protein content was determinate with RD DC Protein Assay Kit (Bio-Rad, CA, USA) and mtDNA content was quantified with Qubit™ fluorometer (Invitrogen, CA, USA).

2.7. Blue-native PAGE separation of mitochondrial membrane complexes and in-gel activity of complexes IV and V

BN-PAGE was performed using the method described by Schagger and von Jagow (Taylor et al., 1994) with minor modifications. Muscle mitochondria (400 μg of protein) from each animal were pelleted by centrifugation at 20,000 *g* for 10 min and then resuspended in solubilization buffer (50 mM NaCl, 50 mM imidazole, 2 mM ε-amino n-caproic acid, 1 mM EDTA pH 7.0) with 1% (w/v) digitonin. After 10 min on ice, insoluble material was removed by centrifugation at 20,000 *g* for 30 min at 4 °C. Soluble components were combined with 0.5% (w/v) Coomassie Blue G250, 50 mM ε-amino n-caproic acid, 4% (w/v) glycerol and separated on a 4–13% gradient acrylamide gradient gel with 3.5% sample gel on top. Anode buffer contained 25 mM imidazole pH 7.0. Cathode buffer (50 mM tricine and 7.5 mM imidazole pH 7.0) containing 0.02% (w/v) Coomassie Blue G250 was used during 1 h at 70 V, the time needed for the dye front reach approximately one-third of the gel. Cathode buffer was then replaced with one containing only 0.002% (w/v) Coomassie Blue G250 and the native complexes were separated at 200 V for 4 h at 4 °C. Gels were stained with Coomassie Colloidal for protein visualization and scanned with Molecular Imager Gel Doc XR+ System (Bio-Rad).

The in-gel activity and histochemical staining assays of complexes IV and V were determined using the methods described by Zerbetto et al. (Simon et al., 2003). Complex IV-specific heme stain in BN-PAGE gels was determined using 10 μL horse heart cytochrome c (5 mM) and 0.5 mg diaminobenzidine (DAB) dissolved in 1 mL 50 mM sodium-phosphate, pH 7.2. The reaction was stopped by 50% (v/v) methanol, 10% (v/v) acetic acid, and the gels were then transferred to water. ATP hydrolysis activity of complex V was analyzed by incubating the native gels with 35 mM Tris, 270 mM glycine buffer, pH 8.3 at 37 °C, that had been supplemented with 14 mM $MgSO_4$, 0.2% (w/v) $Pb(NO_3)_2$, and 8 mM ATP. Lead phosphate precipitation that is proportional to the enzymatic ATP hydrolysis activity, was stopped by 50% (v/v) methanol, and the gels were then transferred to water. Band detection and activity quantification were performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

2.8. Spectrophotometric assay of ATP synthase activity

For spectrophotometric determination of complex V respiratory chain activity mitochondrial fractions and whole *gastrocnemius* muscle were disrupted by a combination of freeze–thawing cycles in hypotonic media (25 mM potassium phosphate, pH 7.2) to allow free access to substrates for all assays (Taylor et al., 1994). ATP synthase activity was measured according to Simon et al. (2003). The phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-color complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as an inhibitor of mitochondrial ATPase activity. A Multiskan GO Microplate Spectrophotometer (Thermo Scientific) was used for respiratory chain complexes activities measurement, which was performed at 30 °C.

2.9. Analysis of proteolytic activity through gelatine zymography

Zymography assays were performed according to Caseiro et al. (2012) with minor modifications. Briefly, a 10% SDS-PAGE separation gel with 0.1% of gelatin was used. Fifteen micrograms of protein from whole *gastrocnemius* muscle or urine samples were incubated on charging buffer (100 mM Tris pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) for 10 min on ice, in a proportion of 1:1 (v/v). After the run, gels were incubated in renaturation buffer (2.5% Triton X-100) for 30 min, with agitation. The zymo gels were changed to a development buffer (50 mM Tris, 5 mM NaCl, 10 mM $CaCl_2$, 1 μm $ZnCl_2$, 0.02% (v/v) Triton X-100, pH 7.4) for more 30 min, followed by an overnight incubation at 37 °C in a new development buffer. For specific inhibition studies zymograms were incubated in a solution containing 10 mM EDTA or 1 mM PMSF. The zymo gels were stained with 0.12% (w/v) Coomassie Blue G250 prepared in 20% methanol, after 1 h fixation in a solution of 10% acetic acid and 40% methanol. Gels were then destained with 25% methanol and scanned with Molecular Imager Gel Doc XR+ System.

2.10. Immunoblotting analysis

Equivalent amounts of serum, mitochondrial or whole *gastrocnemius* muscle proteins of each group were electrophoresed on a 12.5% SDS-PAGE as described by Laemmli (1970). Gels were blotted onto a nitrocellulose membrane (Whatman®, Protan) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol) during 2 h (200 mA). Then, nonspecific binding was blocked with 5% (w/v) dry nonfat milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0 and 0.5% Tween 20). Membranes with serum samples were incubated with primary antibody diluted 1:1000 5% (w/v) nonfat dry milk in TBS-T (anti-CRP, anti-GDF8, anti-IL-6, anti-TNF-α or anti-TWEAK) for 2 h at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-rabbit (1:1000; GE Healthcare). Membranes with mitochondria fractions were incubated with primary antibody diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T (anti-paraplegin, anti-ATPB, anti-LONP1 or anti-mtTFA) for 2 h at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit (GE Healthcare), respectively. Whole *gastrocnemius* muscle membranes were incubated with primary antibody diluted 1:1000 in 5% (w/v) BSA in TBS-T (anti-phospho-Akt, anti-atrogin-1, anti-GDF8, anti-TWEAK, anti-MuRF1, anti-TRAF6, anti-phospho-mTOR, anti-phospho-p70S6K, anti-phospho-4E-BP1 or anti-phospho-Smad3) for 2 h at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-rabbit (GE Healthcare). Immunoreactive bands were detected by enhanced chemiluminescence ECL (Amersham Pharmacia Biotech) according to the manufacturer's procedure and images were recorded using X-ray films (Kodak Biomax Light Film, Sigma, MO, USA). The films were scanned in Molecular Imager Gel Doc XR+ System and analyzed with QuantityOne software. Control for protein loading was confirmed by Ponceau S staining.

For the protein carbonyl derivates assay, a given volume (V) of sample containing 20 μg of protein was derivatized with 2,4-dinitrophenylhydrazine (DNPH). Briefly, the sample was mixed with 1 V of 12% sodium dodecyl sulfate, 2 V of 2 mM DNPH/10% trifluoroacetic acid, followed by 30 min of incubation in the dark, after which 1.5 V of 2 M Tris-base/18.3% of β-mercaptoethanol was added for neutralization. After diluting the derivatized proteins

Table 1
Characterization of the animals used in the study regarding body weight, muscle mass and muscle-to-body weight.

Experimental groups	Body weight (g)	Muscle weight (g)	Muscle/body weight ratio (mg g^{-1})
CONT	309.0 ± 21.1	3.3 ± 0.3	10.5 ± 0.5
BBN	256.6 ± 4.1***	2.9 ± 0.1*	11.2 ± 1.5

Values are expressed as mean ± standard deviation.
* $p < 0.05$ vs CONT.
*** $p < 0.001$ vs CONT.

in TBS to obtain a final concentration of 0.001 μg/μL, a 100 μL volume was slot-blotted into a nitrocellulose membrane. For 3-nitrotyrosine, mitochondria and whole *gastrocnemius* muscles samples were diluted in TBS to obtain a final protein concentration of 0.001 μg/μL and a volume of 100 μL was slot-blotted into a nitrocellulose membrane. For MMP-9, whole *gastrocnemius* muscle samples were diluted in TBS to obtain a final protein concentration of 0.001 μg/μL and a volume of 100 μL was slot-blotted into a nitrocellulose membrane.

The slot-blot membranes were blocked with 5% (w/v) dry nonfat milk in TBS-T for 30 min and then incubated for 30 min with primary antibody (anti-MMP-9, anti-3-nitrotyrosine or anti-DNP) diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T. The membranes were washed three times (10 min each) with TBS-T and incubated for 30 min with a solution of horseradish-conjugated anti-mouse or anti-rabbit antibody, respectively, in a dilution of 1:1000. Detection was carried out with enhanced chemiluminescence and images were recorded using X-ray films. The films were scanned in Molecular Imager Gel Doc XR+ System and analyzed with QuantityOne software. Control for protein loading was confirmed by Ponceau S staining.

2.11. Statistical analysis

The results are presented as mean ± SD for each experimental group. The Kolmogorov–Smirnov test was used to test normality of distribution for all data. Since all variable were normally distributed, significant differences between the two groups were evaluated with the unpaired Student's *t*-test. Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA, version 12.0) was used for all analyses. A p value <0.05 was considered significant.

3. Results

3.1. Characterization of rat's response to BBN administration

Body and *gastrocnemius* muscle weight and *gastrocnemius* muscle-to-body weight ratio for the animal groups studied are reported in Table 1. Significant 17% loss of body weight was observed in BBN-treated rats ($p < 0.001$) when compared with control ones, highlighting the cachexia condition. This body weight decrease was accompanied by a 12% reduction in *gastrocnemius* muscle mass ($p < 0.05$ vs CONT). No significant differences of muscle-to-body weight ratio were observed between groups. Moreover, no significant differences in food intake were associated to urothelial carcinoma (Fig. S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.04.014.

3.2. Serum catabolic drive

As can be observed in Table 2, serum markers of inflammation and catabolism presented significant alterations in BBN-treated rats, with a decrease of serum albumin and total protein levels ($p < 0.05$), as well as triglycerides and increase in serum lactate ($p < 0.0001$). Total cholesterol and HDL-cholesterol were not affected by BBN treatment.

These alterations were paralleled by an increase in the levels of the pro-inflammatory cytokines TNF-α ($p < 0.05$), TWEAK and IL-1β ($p < 0.01$), of the acute reaction phase protein CRP ($p < 0.001$) and of the TGF-β member myostatin ($p < 0.01$), assessed by immunoblotting and ELISA (for IL-1β) (Fig. 1). No BBN-related differences of IL-6 were noticed (Fig. 1C). Altogether, data highlight the catabolic profile underlying BBN chemically-induced urothelial carcinoma.

3.3. Urine proteolytic profile

The effect of BBN treatment on urine proteolytic profile was evaluated by zymography with gelatin as substrate. As can be seen in Fig. 2, no differences were observed in the profile of zymo bands but BBN-related alterations in the proteolytic activity were noticed, which were higher for bands 4 and 6. The inhibitory effect of EDTA on zymo proteolytic activity suggests a high contribution of MMPs to the urine proteolytic activity. Considering the molecular weight of the bands with proteolytic activity (according to Uniprot (http://www.uniprot.org/) and Brenda (http://www.brenda-enzymes.info/)) we might suspect of the presence of MMP-2 (72 kDa) and MMP-7 (30 kDa). Since several MMPs present a molecular weight of approximately 50 kDa (e.g. MMP-8, MMP-12 or MMP-16), no association with a specific MMP can be established for band 2.

3.4. Macroscopic and microscopic evaluation of urothelial lesions

The incidence of histopathological lesions in each group is shown in Table 3. No histopathological changes in the urothelium were observed in control rats. Nodular hyperplasia was detected in all bladders of BBN-treated rats whereas simple hyperplasia and dysplasia were observed in more than 75% of the animals. Besides benign lesions, BBN administration induced malignant injury in different areas of the urothelium, which occurred simultaneously in most cases. Signs of invasive carcinoma were only observed in

Table 2
Effect of BBN administration in serum biochemical profile.

Experimental groups	Albumin (g L^{-1})	Protein (g L^{-1})	Lactate (mg dL^{-1})	Cholesterol (mg dL^{-1})	HDL-c (mg dL^{-1})	Triglycerides (mg dL^{-1})
CONT	40.3 ± 3.2	66.5 ± 4.8	49.7 ± 6.45	42.4 ± 16.5	15.3 ± 3.7	94.0 ± 20.0
BBN	36.7 ± 3.0*	62.6 ± 3.6*	92.6 ± 22.4****	30.9 ± 12.2	12.8 ± 2.6	50.0 ± 15.8****

Values are expressed as mean ± standard deviation.
* $p < 0.05$ vs CONT.
**** $p < 0.0001$ vs CONT.

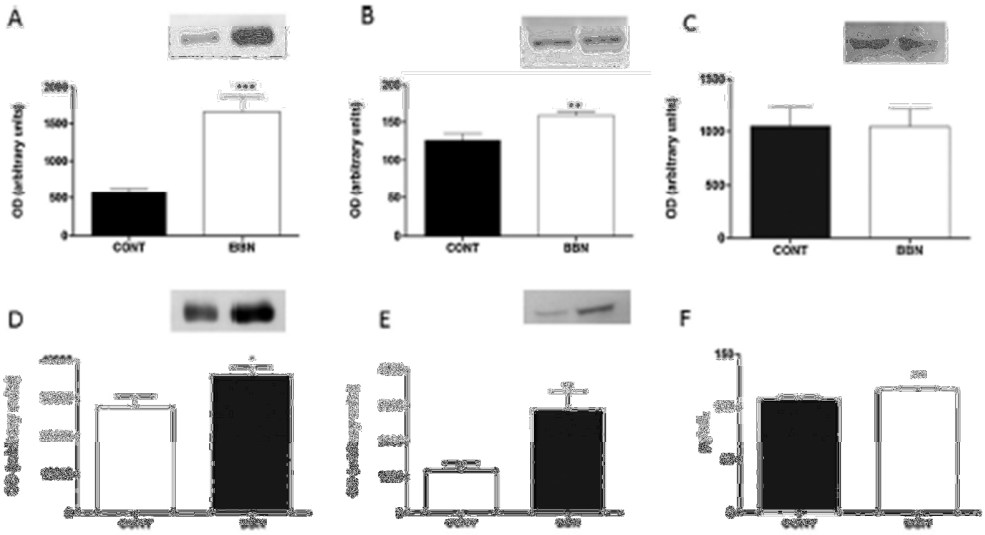

Fig. 1. Serum CRP (A), myostatin (B), IL-6 (C), TNF-α (D) and TWEAK (E) expression evaluated by western blotting, and IL-1β (F) determined by ELISA from BBN and control animals. Representative immunoblots are presented above the corresponding graph. Values are expressed as mean ± standard deviation (*$p < 0.05$ vs CONT, **$p < 0.01$ vs CONT, ***$p < 0.001$ vs CONT).

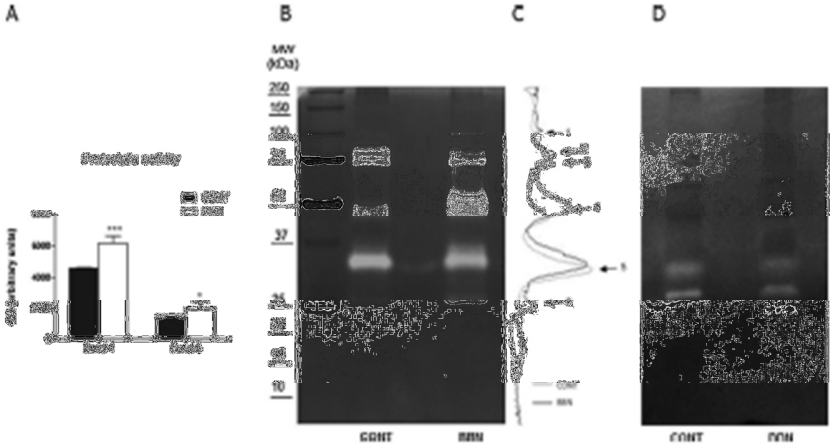

Fig. 2. Effect of BBN administration on urinary proteolytic activity. (B) Representative image of the zymo gel evidencing six bands with proteolytic activity. An overlap of densitometric variation for CONT and BBN lanes is presented in the left side of zymo gel (C). The semi-quantitative analysis of bands 4 and 6 proteolytic activities for each group is presented in (A). Representative image of the zymo gels in the presence of the metalloproteases inhibitor EDTA (D).

Table 3
Incidence of urothelial lesions in Wistar rats exposed to BBN.

Histological lesion	Experimental groups	
	CONT	BBN
Normal urothelium	10/10 (100%)	0 (0%)
Simple hyperplasia	0 (0%)	11/13 (85%)
Nodular hyperplasia	0 (0%)	13/13 (100)
Papillary hyperplasia	0 (0%)	9/13 (70%)
Dysplasia	0 (0%)	10/13 (76.9%)
CIS	0 (0%)	0 (0%)
Papilloma	0 (0%)	3/13 (23%)
Papillary neoplasm of low malignant potential	0 (0%)	8/13 (61.5%)
Low-level papillary carcinoma	0 (0%)	11/13 (84.6%)
High-level papillary carcinoma	0 (0%)	10/13 (76.9%)
Invasive carcinoma	0 (0%)	5/13 (38.5%)
Epidermoid metaplasia	0 (0%)	9/13 (70%)

39% of BBN animals, suggesting a predominance of non-muscle-invasive bladder tumors.

3.5. Analysis of the anabolic/catabolic balance in gastrocnemius muscle

To determine the effect of BBN administration in activated Akt, phopho-Akt levels were evaluated by western blotting. As can be seen in Fig. 3A, a statistically significant decrease in the expression levels of Akt Ser^{473} phosphorylation were noticed in *gastrocnemius* muscle from BBN-treated rats compared to control ones ($p < 0.05$). The expression of mTOR phosphorylated form was also analyzed and a significant decrease ($p < 0.05$) was found on phosphorylated Ser^{2448} mTOR in BBN group (Fig. 3B). The downstream targets of mTOR were also analyzed and lower levels of phosphorylated

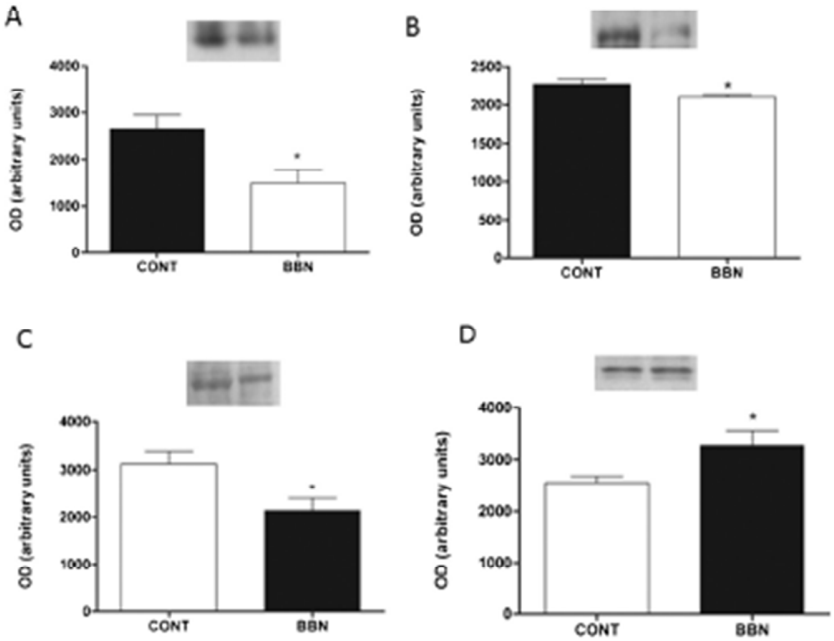

Fig. 3. p-Akt (A), p-mTOR (B), p-S6K1 (C) and p-4EBP1 (D) expression evaluated by western blotting in whole *gastrocnemius* muscle from BBN and control animals. Representative immunoblots are presented above the corresponding graph. Values are expressed as mean ± standard deviation (*$p < 0.05$ vs CONT).

Thr^{389} 70S6K1 were detected but an unexpected higher content of $Thr^{37/46}$ 4E-BP1 was observed in the *gastrocnemius* from rats with urothelial carcinoma (Fig. 3C and D, respectively).

The contribution of the ubiquitin–proteasome system to bladder cancer-induced muscle wasting was evaluated by western blotting analysis of MAFbx/atrogin-1, MuRF-1 and TRAF6 (Fig. 4). Data evidences a statistically significant increase of the E3 ligases atrogin-1 (approximately 11%) and TRAF6 (41%) in the *gastrocnemius* muscle from BBN-treated rats ($p < 0.01$). No differences were noticed for MuRF1 (Fig. 4E). The levels of phosphorylated Ser^{423}/Ser^{425} Smad3 increased in the skeletal muscle of BBN animals, as well as the TGF-β superfamily member myostatin (Fig. 4F and A, respectively; $p < 0.01$). Western blotting analysis of TWEAK expression in whole muscle (Fig. 4B) also evidenced a significant increased content of

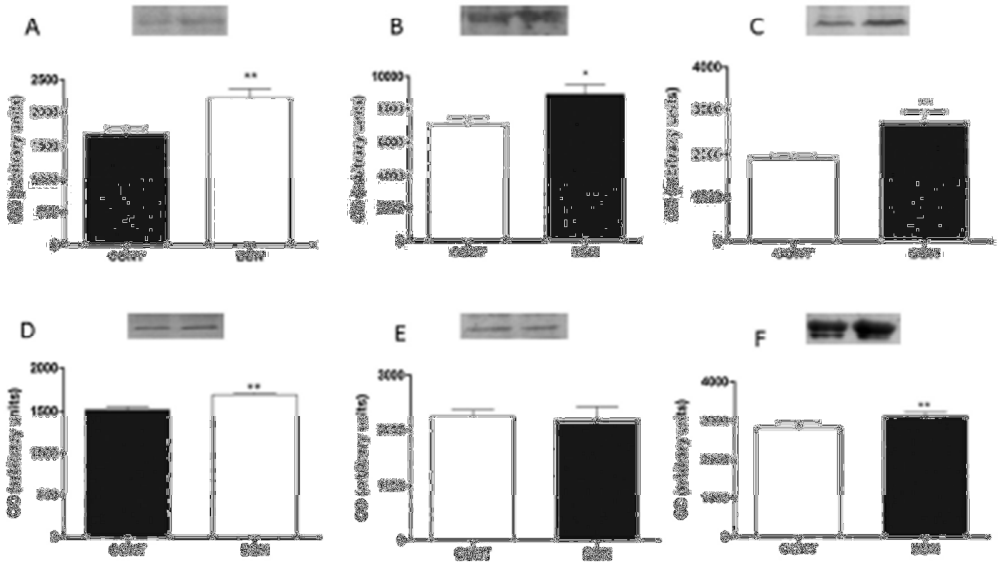

Fig. 4. Myostatin (A), TWEAK (B), TRAF6 (C), atrogin-1 (D), MuRF-1 (E) and p-Smad3 (F) expression evaluated by western blotting in whole *gastrocnemius* muscle from BBN and control animals. Representative immunoblots are presented above the corresponding graph. Values are expressed as mean ± standard deviation (*$p < 0.05$ vs CONT; **$p < 0.01$ vs CONT).

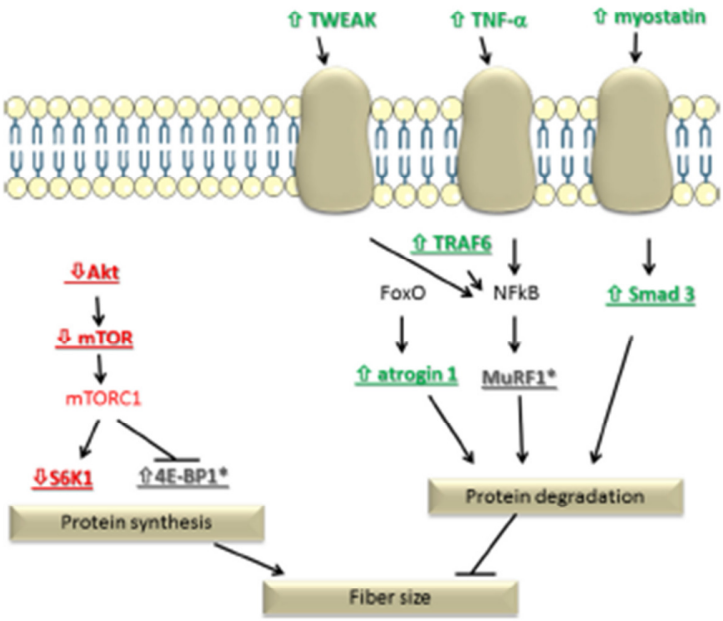

Fig. 5. Integrated perspective of signaling pathways modulated by urothelial carcinoma in gastrocnemius muscle. The proteins analyzed in the present study are highlighted (bold and underlined) with the up-regulated ones presented in green and the down-regulated in red. Proteins with unexpected expression variation are presented in grey and marked with an *.

Figure was produced using Servier Medical Art.

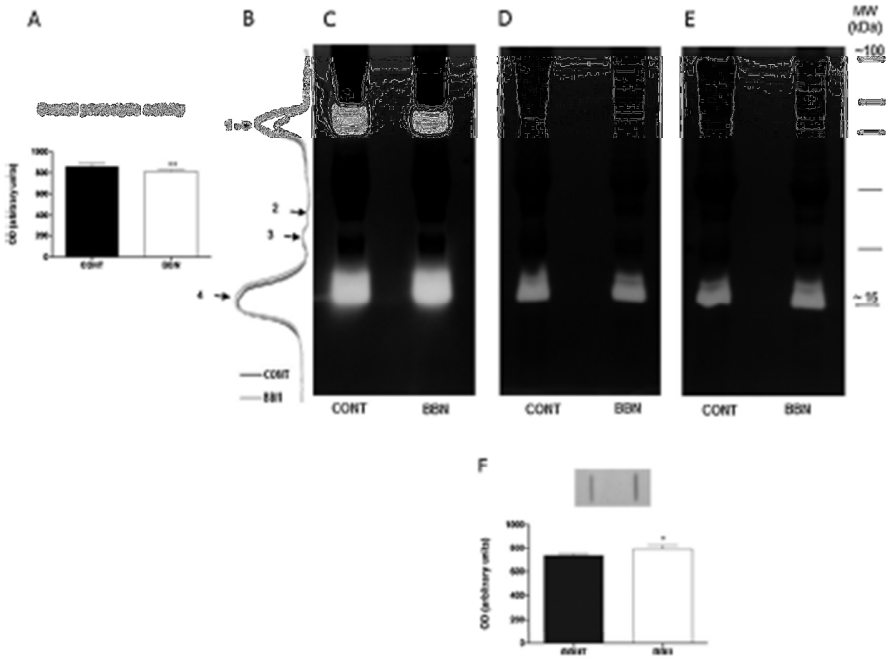

Fig. 6. Effect of BBN administration on whole gastrocnemius muscle proteolytic activity. (C) Representative image of the zymography evidencing two bands with noticeable proteolytic activity (bands 1 and 4). An overlap of densitometric variation for CONT and BBN lanes is presented in the left side of zymo gel (B). Semi-quantitative analysis of overall proteolytic activity for each group is presented in (A). Representative images of the zymo gels in the presence of inhibitors for (D) serine protease (PMSF) and for (E) metalloproteases (EDTA). Comparative analysis of MMP-9 expression between groups is presented in (F). A representative immunoblot is presented above the graph. Values are expressed as mean ± standard deviation (*$p < 0.05$ vs CONT; **$p < 0.01$ vs CONT).

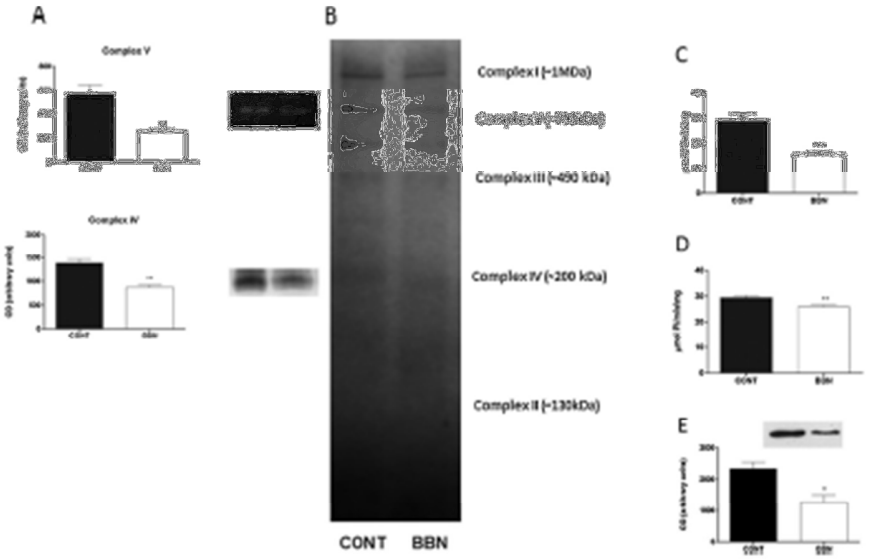

Fig. 7. Evaluation of respiratory chain structural organization and activity. (A) Representative image of histochemical staining of in-gel activity of complexes IV and V. On the left side is presented a semi-quantitative analysis of in-gel activity of both complexes. (B) Representative image of supramolecular architecture of mitochondrial membrane complexes resolved by BN-PAGE for BBN and CONT groups. ATP synthase activity on mitochondria (C) and on whole muscle (D) spectrophotometrically assessed and ATP synthase β (E) expression evaluated by western blotting. Representative immunoblot is presented above the graph. Values are expressed as mean ± standard deviation ($^{*}p < 0.05$ vs CONT; $^{**}p < 0.01$ vs CONT; $^{***}p < 0.001$ vs CONT).

this pro-inflammatory cytokine in BBN-treated animal ($p < 0.01$). To better visualize all these data, an integrative picture is presented in Fig. 5.

The analysis of muscle proteolysis performed by gelatin zymography evidenced an overall 5% decrease in BBN-treated rats ($p < 0.01$; Fig. 6A). The densitometric evaluation of the zymogram profile (Fig. 6B) displayed four common bands in the muscle of all animals. Two of these bands showed higher activity, one with approximately 80 kDa (band 1) and other with approximately 15 kDa (band 4). Band 1 activity was the most affected by BBN-related urothelial carcinoma. To have a general idea of protease classes present in each band, zymo gels were incubated with different inhibitors at their maximum effective concentration: PMSF (serine protease inhibitor) and EDTA (metalloproteinase inhibitor). Data obtained (Fig. 6D and E) suggest the involvement of both serine- and metalloproteases. The slot blot analysis of MMP-9 showed a significantly higher expression of this metalloprotease in BBN-treated rats (7.5% ($p < 0.05$); Fig. 6F).

3.6. Analysts of mitochondrial functionality, proteolysis and protein susceptibility to oxidation

The levels of mtDNA and mitochondrial protein were quantified in mitochondria isolated from *gastrocnemius* muscle. As can be depicted from Table 4, a higher concentration of muscle mtDNA and protein-to-muscle mass ratio were noticed in BBN-treated rats ($p < 0.05$); however, no significant differences in the ratio mtDNA-to-nDNA and on the SDS-PAGE quantitative profile (Fig. S2) were noticed among groups. Taken together, data suggest the occurrence of muscle atrophy in the animals with urothelial carcinoma. Moreover, these mitochondria showed lower ability to produce ATP in view of lower cytochrome c oxidase and ATP synthase in-gel activities, further corroborated by the spectrophotometric determination of complex V activity and ATP synthase beta expression (Fig. 7A and C). Though lower ATP synthase activities were observed either in isolated mitochondria (Fig. 7C) as in whole muscle (Fig. 7D) of BBN animals, a higher BBN-related decrease was noticed in isolated mitochondria. Lower mtTFA expression levels were also observed in the skeletal muscle mitochondria from these animals (Fig. 8C).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.04.014.

To evaluate the involvement of the proteases known to be implicated in mitochondrial protein quality control (PQC), we analyzed the expression of the specific m-AAA protease paraplegin and of the matrix Lon protease (Fig. 8A and B). Lower expression values of these serine proteases (75% for paraplegin ($p < 0.05$; Fig. 8A) and 40% for Lon ($p < 0.001$; Fig. 8B)) were observed in BBN-treated animals.

Taken in consideration the involvement of PQC proteases in the elimination of damaged proteins and their impairment in BBN

Table 4
Effect of BBN administration in mitochondrial protein and mtDNA content, and in the ratio mtDNA-to-nDNA in *gastrocnemius* muscle.

Experimental groups	Protein/mtDNA ($\mu g\,g^{-1}$)	mtDNA/muscle weight ($\mu g\,g^{-1}$)	Protein/muscle weight ($mg\,g^{-1}$)	mtDNA/nDNA (%)
CONT	1.8 ± 0.8	1.1 ± 0.2	1995.9 ± 672.3	21.6 ± 5.6
BBN	0.8 ± 0.3*	4.3 ± 2.8*	2875.1 ± 759.3*	18.7 ± 5.7

Values are expressed as mean ± standard deviation.
* $p < 0.05$ vs CONT.

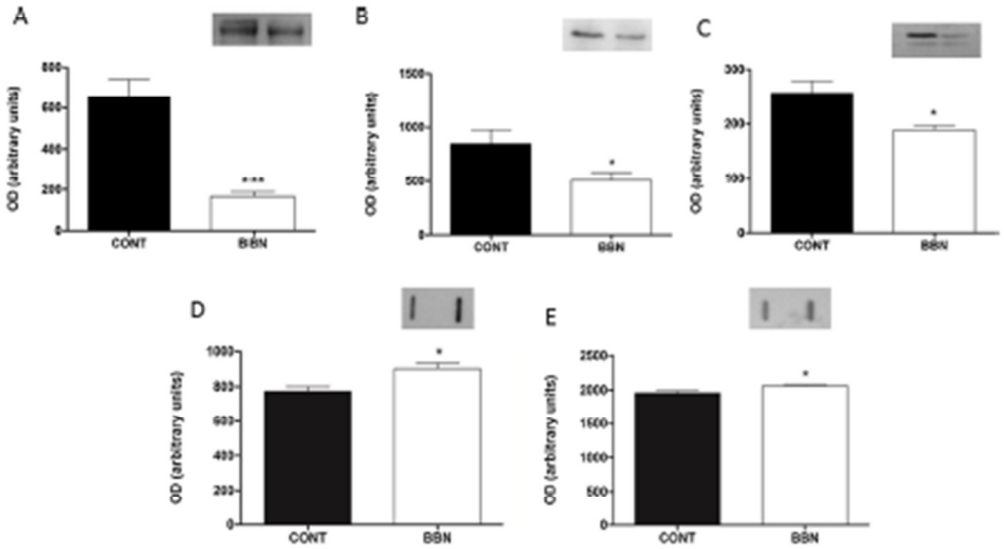

Fig. 8. Effect of BBN administration on m-AAA paraplegin (A) and LonP1 (B), and mtTFA (C) expression evaluated by western blotting in mitochondrial fractions. Protein carbonyl (D) and 3-nitrotyrosine (E) content evaluated by slot blot in isolated mitochondria from BBN and CONT animals. Representative immunoblots are presented above the corresponding graph. Values are expressed as mean ± standard deviation (*$p < 0.05$ vs CONT; ***$p < 0.001$ vs CONT)

mitochondria, the levels of carbonylated proteins were measured by slot blot after derivatization with dinitrophenylhydrazine. A significant increase of 16% ($p < 0.05$) was observed in mitochondria from BBN animals' skeletal muscle when compared with controls (Fig. 8D). In order to assess the protein nitration levels, we measured 3-nitrotyrosine content and, as can be seen in Fig. 8E, higher levels of nitrated proteins were observed in mitochondria from BBN-treated rats (5% ($p < 0.05$)).

4. Discussion

4.1. Overall findings

In the present study, we utilized an animal model of urothelial carcinogenesis chemically induced by the administration of N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN), a genotoxic compound known to induce cancer (Oliveira et al., 2006). After administration of BBN in the drinking water, bladder tumors formed within 20 weeks. These tumors in rats are equivalent to the non-muscle-invasive urothelial carcinoma clinically observed in humans (Palmeira et al., 2009, 2010; Oliveira et al., 2007). This well-established model reflects one of the most commonly occurring malignant tumors of the urinary tract, associated with chemical risk factors as smoking and aromatic amines (Oliveira et al., 2007; Roupret et al., 2011). Though it is recognized that carcinogen-induced tumors resemble human cancer since they arise spontaneously and reproduce tumor–host interactions, they are not generally used for the study of cancer-induced muscle wasting since the experiments tend to be lengthy and costly (Bennani-Baiti and Walsh, 2011). At the end of the protocol, most of the animals showed histologic signs of non-muscle-invasive carcinoma (Table 3) and evidenced 17% loss of body weight, a value that in humans is seen as a sign of moderate cachexia according to the cachexia score (CASCO) (Argiles et al., 2011). This body weight decrease was paralleled by a 12% loss of *gastrocnemius* mass (Table 1), suggestive of altered muscle properties such as weakness and fatigue (Shum et al., 2012; Argiles et al., 2011).

4.2. Catabolic profile associated with carcinoma-induced muscle wasting

The significantly high serum levels of the pro-inflammatory cytokines IL-1β, TNF-α and TWEAK, and of the acute phase protein CRP observed in BBN animals, supports the catabolic profile noticed in this group. Despite evidences of systemic inflammation, no significant effects on animals' appetite were noticed. No differences of serum IL-6 levels were observed; however, the association between serum levels of this cytokine and weight loss is not straightforward and in studies performed with mice bearing clones of the colon-26 tumor or with patients with non-small-cell-lung cancer no relation between serum IL-6 and cachexia was observed (Patra and Arora, 2012). Myostatin and related ligands have also been suggested as the main responsible for weight loss in patients afflicted by various forms of cancer (Lokireddy et al., 2012). The levels of this member from the transforming growth factor-β superfamily known to be a negative regulator of muscle bulk (Springer et al., 2010) was found in significantly higher levels in the serum of BBN rats (Fig. 1B). Though expressed almost exclusively in skeletal muscle (Springer et al., 2010), myostatin was recently proposed as a novel tumoral factor that might initiate the pathogenesis of cancer cachexia (Lokireddy et al., 2012). Our data suggest that the raise of this TGF-β ligand is due, at least partially, to an increase of its expression in the *gastrocnemius* muscle (Fig. 4A).

BBN administration also induced metabolic disturbances including hypoalbuminaemia and high levels of serum lactate. The diminishing of serum triglycerides does not support the usually described cancer cachexia-related lipolysis (Tisdale, 2009). Nevertheless, bladder cancer-related catabolic phenotype is not always accompanied by altered lipolysis (Lattermann et al., 2003). The higher activity of MMPs in the urine of BBN rats (Fig. 2) further supports the catabolic profile related to urothelial carcinoma. The release of MMP-1, MMP-2 and MMP-9 by rat bladder tumorigenic/invasive cell lines was previously reported (Kawamata et al., 1993). Our data suggest the contribution of MMP-2 and MMP-7 in the extracellular matrix remodeling of urothelium carcinoma. Other MMPs seem involved but further analysis should be

performed to investigate their association with bladder cancer-related muscle wasting. Though interest has been noticed in the monitoring of MMPs in the clinical setting as diagnostic or prognostic biomarkers of bladder cancer (Szarvas et al., 2011), few studies have analyzed the association of these proteases with cancer-induced muscle wasting (Camargo et al., 2011; Peluffo et al., 2004).

Once established the urothelial carcinoma-induced catabolic phenotype, the molecular mechanisms underlying bladder cancer-induced muscle wasting were searched. Myostatin together with cytokines like IL-1β, TNF-α and TWEAK elicits catabolism on skeletal muscle by elevating the levels of muscle-specific E3 ligases and increasing the intracellular protein degradation through the ubiquitin–proteasome pathway (Tisdale, 2009; Lokireddy et al., 2012; Skipworth et al., 2007). Myostatin was reported to block IGF1-PI3K-Akt pathway and to activate FoxO1, promoting the increased expression of atrogin-1, in an NFkB-independent manner. NFkB activation is usually induced by TNF-α and TNF-like weak inducer of apoptosis (TWEAK) leading to muscle wasting mediated by MuRF-1 (Bonaldo and Sandri, 2013; Fearon et al., 2012; Romanello and Sandri, 2013), which levels were unaltered in BBN *gastrocnemius* (Fig. 4E). Another key player in NFkB signaling upregulated in an urothelial carcinoma-dependent way was the ubiquitin ligase TRAF6 that is required not only for the induction of ubiquitin–proteasome system but also of autophagy–lysosome pathway (Bonaldo and Sandri, 2013). Other proteases, namely MMP-9, were found to contribute to muscle proteolysis in BBN-treated rats (Fig. 6). Concomitantly, reduced levels of phosphorylated Akt, mTOR and p70S6K were observed (Fig. 3) suggesting reduced ribosome formation and protein synthesis in *gastrocnemius*, a muscle with a mixed metabolic phenotype. The magnitude of this unbalance between protein synthesis and degradation might differ according to muscle phenotype, as previously suggested for atrophic conditions (Fanzani et al., 2012).

4.3. Mitochondrial-related mechanisms on bladder cancer-induced muscle wasting

Along with muscle catabolism induced by the neoplastic growth-induced systemic disturbance, we detected alterations in mitochondria, a critical regulator of muscle protein turnover (White et al., 2012; Romanello and Sandri, 2010). As hypothesized by Julienne et al. (2012), mitochondria could be the place of energy wasting in situations of high muscle proteolysis as in cancer cachexia. Indeed, data from the present study supports the lower ability to produce ATP in the *gastrocnemius* of BBN-treated animals, taken in consideration the lower cytochrome c oxidase and ATP synthase activities (Fig. 7). These functional alterations were previously related to changes in the normal appearance of mitochondria, namely the presence of electron-lucent areas and swelling, which are indicative of cristae loss and ATP depletion, respectively (Shum et al., 2012). The lower levels of the transcriptional factor mtTFA observed in BBN group (Fig. 8C) might be associated with impaired mitochondria transcription, an important step in mitochondrial biogenesis (Maniura-Weber et al., 2004). These results are in agreement with the previous findings of White et al. (2012), who reported the diminishing of PGC-1α, a marker of mitochondrial biogenesis, and a shift towards smaller mitochondria with the progression of body loss. PGC-1α has been related to mTOR signaling and its reduction in BBN animals points to the repression of the transcription of genes involved in oxidative metabolism (Cunningham et al., 2007). Mitochondrial alterations in skeletal muscle might contribute to the increase in serum lactate concentration associated to bladder cancer supplying whole body energy needs (Julienne et al., 2012). Mitochondrial dysfunction has been related to increased ROS production (Lokireddy et al., 2012). Our data highlight the accumulation of oxidatively modified proteins, either carbonylated or nitrated (Fig. 8), that were not cleared out from BBN mitochondria by the PQC systems. Indeed, mitochondria function depends on the mitochondrial PQC that includes the mitochondrial proteases and the mitochondrial-shaping machinery (Romanello and Sandri, 2013). Mitochondrial proteases are regulators of organelle health, by removing misfolded or oxidatively damaged proteins, maintaining the cellular redox homeostasis (Venkatesh et al., 2012; Bulteau and Bayot, 2011). Data here presented evidence lower levels of the AAA protease paraplegin and the matrix Lon protease in the skeletal muscle mitochondria from BBN-treated rats (Fig. 8). Paraplegin coassembles with a homologous protein, AFG3L2, in the mitochondrial inner membrane and when this complex is lost occurs a decrease of OXPHOS complex I activity and an increased sensitivity to oxidative stress (Atorino et al., 2003). The matrix Lon protease has been suggested to have a similar role to the proteasome in cytosol in eliminating oxidatively modified mitochondrial proteins. This protease also displays chaperone properties and its loss of function was previously related to massive apoptosis, with the classical hallmark of caspase-3 activation, and deregulation of mtDNA replication (Bulteau and Bayot, 2011). Lon-mediated degradation of mtTFA was also reported and seems a PQC mechanism for eliminating misfolded or damaged mtTFA, and/or a regulatory mechanism for controlling mtDNA metabolism (Venkatesh et al., 2012). The severe mitochondrial dysfunction might lead to the activation of mitophagy in order to enable organelle replacement with new, more efficient ones. Recently, Lokireddy et al. (2012), reported a reduction of mtDNA copy number *per* nuDNA in the skeletal muscle from C26-tumor bearing mice and associated it with mitophagy, a process that seems to be mediated by mTOR (Romanello and Sandri, 2013).

In summary, using an animal model of urothelial carcinoma we observed that tumor–host interaction results in increased levels of pro-inflammatory mediators and myostatin, and in higher levels of urinary MMPs, which highlight the catabolic profile underlying body weight loss. Concomitantly with the imbalance between protein synthesis and degradation due to the activation of the ubiquitin–proteasome and suppression of PI3K/Akt/mTOR pathway, we noticed a decrease of the oxidative metabolism in wasted muscle. Focusing on mitochondria isolated from *gastrocnemius* muscle, our data showed that the impairment of mitochondrial PQC systems involving mitochondrial proteases seems to be linked to an increased generation of ROS and with the consequent accumulation of oxidatively modified proteins, which ultimately leads to lower mitochondrial ability to generate ATP. The dynamics and functional versatility of mitochondrial PQC systems need to be further explored aiming to define therapeutic targets to counteract cancer-related muscle wasting.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

This work was supported by Portuguese Foundation for Science and Technology (FCT), European Union, QREN, FEDER and COMPETE for funding the QOPNA research unit (project PEst-C/QUI/UI0062/2011), the research project (PTDC/DES/114122/2009; COMPETE, FCOMP-01-0124-FEDER-014707) and post-graduation students [grant numbers SFRH/BD/66642/2009 (to A.I.P.), SFRH/BPD/90010/2012 (to D.M.G.)].

References

Argiles JM, Lopez-Soriano FJ, Toledo M, Betancourt A, Serpe R, Busquets S. The cachexia score (CASCO): a new tool for staging cachectic cancer patients. Journal of Cachexia, Sarcopenia and Muscle 2011;2:87–93.

Atorino L, Silvestri L, Koppen M, Cassina L, Ballabio A, Marconi R, et al. Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia. The Journal of Cell Biology 2003;163:777–87.

Bennani-Baiti N, Walsh D. Animal models of the cancer anorexia–cachexia syndrome. Supportive Care in Cancer: Official Journal of the Multinational Association of Supportive Care in Cancer 2011;19:1451–63.

Blum D, Omlin A, Baracos VE, Solheim TS, Tan BH, Stone P, et al. Cancer cachexia: a systematic literature review of items and domains associated with involuntary weight loss in cancer. Critical Reviews in Oncology/Hematology 2011;80:114–44.

Bonaldo P, Sandri M. Cellular and molecular mechanisms of muscle atrophy. Disease Models & Mechanisms 2013;6:25–39.

Bonetto A, Aydogdu T, Kunzevitzky N, Guttridge DC, Khuri S, Koniaris LG, et al. STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia. PLoS One 2011;6:e22538.

Bulteau AL, Bayot A. Mitochondrial proteases and cancer. Bioenergetics 2011;1807:595–601.

Buskermolen S, Langius JA, Kruizenga HM, Ligthart-Melis GC, Heymans MW, Verheul HM. Weight loss of 5% or more predicts loss of fat-free mass during palliative chemotherapy in patients with advanced cancer: a pilot study. Nutrition and Cancer 2012;64(6):826–32.

Camargo CA, da Silva ME, da Silva RA, Justo GZ, Gomes-Marcondes MC, Aoyama H. Inhibition of tumor growth by quercetin with increase of survival and prevention of cachexia in Walker 256 tumor-bearing rats. Biochemical and Biophysical Research Communications 2011;406:638–42.

Caseiro A, Vitorino R, Barros AS, Ferreira R, Calheiros-Lobo MJ, Carvalho D, et al. Salivary peptidome in type 1 diabetes mellitus. Biomedical Chromatography 2012;26:571–82.

Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature 2007;450:736–40.

Dempsey DT, Buzby GP, Mullen JL. Nutritional assessment in the seriously ill patient. Journal of the American College of Nutrition 1983;2:15–22.

Epstein JI, Amin MB, Reuter VR, Mostofi FK. The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. American Journal of Surgical Pathology 1998;22:1435–48.

Fanzani A, Conraads VM, Penna F, Martinet W. Molecular and cellular mechanisms of skeletal muscle atrophy: an update. Journal of Cachexia, Sarcopenia and Muscle 2012;3:163–79.

Fearon KC, Glass DJ, Guttridge DC. Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metabolism 2012;16:153–66.

Jacobs BL, Montgomery JS, Zhang Y, Skolarus TA, Weizer AZ, Hollenbeck BK. Disparities in bladder cancer. Urologic Oncology 2012;30:81–8.

Julienne CM, Dumas JF, Goupille C, Pinault M, Berri C, Collin A, et al. Cancer cachexia is associated with a decrease in skeletal muscle mitochondrial oxidative capacities without alteration of ATP production efficiency. Journal of Cachexia, Sarcopenia and Muscle 2012;3(4):265–75.

Kawamata H, Kameyama S, Nan L, Kawai K, Oyasu R. Effect of epidermal growth factor and transforming growth factor beta 1 on growth and invasive potentials of newly established rat bladder carcinoma cell lines. International Journal of Cancer 1993;55:968–73.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

Lattermann R, Geisser W, Georgieff M, Wachter U, Goertz A, Gnann R, et al. Integrated analysis of glucose, lipid, and urea metabolism in patients with bladder cancer: impact of tumor stage. Nutrition 2003;19:589–92.

Lokireddy S, Wijesoma IW, Bonala S, Wei M, Sze SK, McFarlane C, et al. Myostatin is a novel tumoral factor that induces cancer cachexia. The Biochemical Journal 2012;446(1):23–36.

Maniura-Weber K, Goffart S, Garstka HL, Montoya J, Wiesner RJ. Transient overexpression of mitochondrial transcription factor A (TFAM) is sufficient to stimulate mitochondrial DNA transcription, but not sufficient to increase mtDNA copy number in cultured cells. Nucleic Acids Research 2004;32:6015–27.

Mistry M, Parkin DM, Ahmad AS, Sasieni P. Cancer incidence in the United Kingdom: projections to the year 2030. British Journal of Cancer 2011;105:1795–803.

Oliveira PA, Palmeira C, Colaco A, de la Cruz LF, Lopes C. DNA content analysis, expression of Ki-67 and p53 in rat urothelial lesions induced by N-butyl-N-(4-hydroxybutyl) nitrosamine and treated with mitomycin C and bacillus Calmette-Guerin. Anticancer Research 2006;26:2995–3004.

Oliveira PA, Adega F, Palmeira CA, Chaves RM, Colaco AA, Guedes-Pinto H, et al. DNA study of bladder papillary tumours chemically induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in Fisher rats. International Journal of Experimental Pathology 2007;88:39–46.

Palmeira C, Oliveira PA, Arantes-Rodrigues R, Colaco A, De la Cruz PL, Lopes C, et al. DNA cytometry and kinetics of rat urothelial lesions during chemical carcinogenesis. Oncology Reports 2009;21:247–52.

Palmeira C, Oliveira PA, Lameiras C, Amaro T, Silva VM, Lopes C, et al. Biological similarities between murine chemical-induced and natural human bladder carcinogenesis. Oncology Letters 2010;1:373–7.

Patra SK, Arora S. Integrative role of neuropeptides and cytokines in cancer anorexia–cachexia syndrome. Clinica Chimica Acta: International Journal of Clinical Chemistry 2012;413:1025–34.

Peluffo GD, Stillitani I, Rodriguez VA, Diament MJ, Klein SM. Reduction of tumor progression and paraneoplastic syndrome development in murine lung adenocarcinoma by nonsteroidal antiinflammatory drugs. International Journal of Cancer 2004;110:825–30.

Romanello V, Sandri M. Mitochondrial biogenesis and fragmentation as regulators of muscle protein degradation. Current Hypertension Reports 2010;12:433–9.

Romanello V, Sandri M. Mitochondrial biogenesis and fragmentation as regulators of protein degradation in striated muscles. Journal of Molecular and Cellular Cardiology 2013;55:64–72.

Roupret M, Zigeuner R, Palou J, Boehle A, Kaasinen E, Sylvester R, et al. European guidelines for the diagnosis and management of upper urinary tract urothelial cell carcinomas: 2011 update. European Urology 2011;59:584–94.

Shum AM, Mahendradatta T, Taylor RJ, Painter AB, Moore MM, Tsoli M, et al. Disruption of MEF2C signaling and loss of sarcomeric and mitochondrial integrity in cancer-induced skeletal muscle wasting. Aging 2012;4:133–43.

Simon N, Papa K, Vidal J, Boulamery A, Bruguerolle B. Circadian rhythms of oxidative phosphorylation: effects of rotenone and melatonin on isolated rat brain mitochondria. Chronobiology International 2003;20:451–61.

Skipworth RJ, Stewart GD, Dejong CH, Preston T, Fearon KC. Pathophysiology of cancer cachexia: much more than host–tumour interaction? Clinical Nutrition 2007;26:667–76.

Springer J, Adams V, Anker SD, Myostatin. Regulator of muscle wasting in heart failure and treatment target for cardiac cachexia. Circulation 2010;121:354–6.

Szarvas T, vom Dorp F, Ergun S, Rubben H. Matrix metalloproteinases and their clinical relevance in urinary bladder cancer. Nature Reviews Urology 2011;8:241–54.

Taylor RW, Birch-Machin MA, Bartlett K, Lowerson SA, Turnbull DM. The control of mitochondrial oxidations by complex III in rat muscle and liver mitochondria. Implications for our understanding of mitochondrial cytopathies in man. The Journal of Biological Chemistry 1994;269:3523–8.

Tisdale MJ. Mechanisms of cancer cachexia. Physiological Reviews 2009;89:381–410.

Tonkonogi M, Sahlin K. Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. Acta Physiologica Scandinavica 1997;161:345–53.

Venkatesh S, Lee J, Singh K, Lee I, Suzuki CK. Multitasking in the mitochondrion by the ATP-dependent Lon protease. Biochimica et Biophysica Acta 2012;1823:56–66.

Wang X, Pickrell AM, Zimmers TA, Moraes CT. Increase in muscle mitochondrial biogenesis does not prevent muscle loss but increased tumor size in a mouse model of acute cancer-induced cachexia. PLoS One 2012;7:e33426.

White JP, Baynes JW, Welle SL, Kostek MC, Matesic LE, Sato S, et al. The regulation of skeletal muscle protein turnover during the progression of cancer cachexia in the Apc(Min/+) mouse. PLoS One 2011;6:e24650.

White JP, Puppa MJ, Sato S, Gao S, Price RL, Baynes JW, et al. IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse. Skeletal Muscle 2012;2:14.

Zhou X, Wang JL, Lu J, Song Y, Kwak KS, Jiao Q, et al. Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. Cell 2010;142:531–43.

3.1.2 Paper III

Overexpression of tumour-associated carbohydrate antigen Sialyl-Tn in advanced bladder tumours.

Ferreira JA, Videira Pa, Lima L, Pereira S, Silva M, Carrascal M, Severino PF, Fernandes E, Almeida A, **Costa C**, Vitorino R, Amaro T, Oliveira MJ, Reis CA, Dall'Olio F, Amado F, Santos LL.

Mol Oncol. 2013. Jun;7(3):719-31. doi: 10.1016/j.molonc.2013.03.001.

MOLECULAR ONCOLOGY 7 (2013) 719–731

available at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/molonc

Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours

José Alexandre Ferreira[a,b,*,1], Paula A. Videira[c,**,1], Luís Lima[b,d,e,f], Sofia Pereira[b,g], Mariana Silva[c], Mylène Carrascal[c], Paulo F. Severino[c,h], Elisabete Fernandes[b], Andreia Almeida[a,b], Céu Costa[b,g], Rui Vitorino[a], Teresina Amaro[i], Maria J. Oliveira[j,k,l], Celso A. Reis[d,k,m], Fabio Dall'Olio[h], Francisco Amado[a,n], Lúcio Lara Santos[b,g,o]

[a]QOPNA, Mass Spectrometry Center, Department of Chemistry, University of Aveiro, Aveiro, Portugal
[b]Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Porto, Portugal
[c]CEDOC, Departamento de Imunologia, Faculdade de Ciências Médicas, FCM, Universidade Nova de Lisboa, Lisboa, Portugal
[d]Institute of Biomedical Sciences of Abel Salazar, University of Porto, Porto, Portugal
[e]Nucleo de Investigação em Farmácia – Centro de Investigação em Saúde e Ambiente (CISA), Health School of the Polytechnic Institute of Porto, Porto, Portugal
[f]LPCC, Research Department-Portuguese League Against Cancer (NRNorte), Portugal
[g]Health School of University of Fernando Pessoa, Porto, Portugal
[h]Department of Experimental, Clinical and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy
[i]Department of Anatomic Pathology, Hospital Pedro Hispano, Matosinhos, Portugal
[j]INEB – Institute of Biomedical Engineering, Porto University, Portugal
[k]Department of Pathology e Oncology, Faculty of Medicine, Porto University, Portugal
[l]Department of Biology, Faculty of Sciences, Porto University, Portugal
[m]Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal
[n]School of Health Sciences, University of Aveiro (ESSUA), Portugal
[o]Department of Surgical Oncology, Portuguese Institute of Oncology, Porto, Portugal

ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form
11 March 2013
Accepted 12 March 2013
Available online 21 March 2013

Keywords:
Sialyl-Tn
Bladder cancer
Glycosylation
ST6GalNAc.I

ABSTRACT

Little is known on the expression of the tumour-associated carbohydrate antigen sialyl-Tn (STn), in bladder cancer. We report here that 75% of the high-grade bladder tumours, presenting elevated proliferation rates and high risk of recurrence/progression expressed STn. However, it was mainly found in non-proliferative areas of the tumour, namely in cells invading the basal and muscle layers. STn was also found in tumour-adjacent mucosa, which suggests its dependence on a field effect of the tumour. Furthermore, it was not expressed by the normal urothelium, demonstrating the cancer-specific nature of this antigen. STn expression correlated with that of sialyltransferase ST6GalNAc.I, its major biosynthetic enzyme. The stable expression of ST6GalNAc.I in the bladder cancer cell line MCR induced STn expression and a concomitant increase of cell motility and invasive capability. Altogether, these results indicate for the first time a link between STn

* Corresponding author. QOPNA, Mass Spectrometry Center, Department of Chemistry, University of Aveiro, Aveiro, Portugal.
** Corresponding author.
E-mail addresses: alexandrecastroferreira@gmail.com, jferreira@dq.ua.pt (J.A. Ferreira), paula.videira@fcm.unl.pt (P.A. Videira).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.molonc.2013.03.001

Tumour-associated glycans
Proliferative bladder cancer

expression and malignancy in bladder cancer. Hence, therapies targeting STn may constitute new treatment approaches for these tumours.

1. Introduction

Bladder cancer, the fifth most common cancer in Western society, is a growing concern, owing to increased incidence during the past years (Ploeg et al., 2009; van Rhijn et al., 2009). Most of the newly diagnosed bladder cancer cases are superficial, or low-grade non-muscle invasive papillary tumours, being conservatively treated by complete transurethral resection of the tumour (Babjuk et al., 2012). However, approximately half of the patients show a high-percentage of recurrences and an elevated risk of progression to muscle invasive disease, which correlates with poor prognosis (Hussain et al., 2009). The risk of recurrence and/or progression is mostly determined by clinicopathological features (Babjuk et al., 2012). According to the European Organization for Research and Treatment of Cancer (EORTC), this group includes high grade (HG) papillary tumours and carcinoma *in situ* (CIS) and those with multifocal or recurrent lesions (Babjuk et al., 2012). The evaluation of the nuclear protein Ki-67 (Ki-67 proliferation index), an established marker of cell proliferation, is often used to enhance the prognostic accuracy of risk classification given by clinicopathological features (Margulis et al., 2009; Santos et al., 2003), since it is considered a surrogate biomarker of bladder cancer aggressiveness, disease recurrence and progression (Margulis et al., 2009; Santos et al., 2003).

Tumour resection followed by a schedule of intravesical instillations with live attenuated strains of *Mycobacterium bovis* (Bacillus Calmette–Guérin, BCG) is the standard adjuvant therapeutic option for high-risk of recurrence/progression bladder tumours (Askeland et al., 2012; Babjuk et al., 2012). Although BCG has improved the management of high-risk patients, 30–40% of cases either show intolerance or relapse after treatment (Yates and Roupret, 2011). Consequently, these patients require life-long follow-up and repeated courses of treatment making bladder cancer the costliest to treat among solid tumours (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Upon therapeutic failure and/or muscle invasion, cystectomy is advocated for oncological control (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Furthermore, 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 (Dovedi and Davies, 2009).

Vaccines using tumour-associated glycans, in association with immunological boosters, are emerging as potential therapeutic strategies against cancer (Hakomori, 2001; Lakshminarayanan et al., 2012; Ryan et al., 2010; Sorensen et al., 2006). In the forefront of these antigens is sialyl-Tn (STn; Neu5Acα2-6GalNAcα-O-Ser/Thr) (Gilewski et al., 2007; Julien et al., 2009; Miles et al., 2011). STn has been mostly observed in tumour-associated mucins due to their high number of potential *O*-glycosylation sites (Clement et al., 2004; Conze et al., 2010; Julien et al., 2006; Marcos et al., 2011; Pinto et al., 2012). However, integrins (Clement et al., 2004) and CD44 (Julien et al., 2006), among other proteins, may also carry this posttranslational modification. Overexpression of STn antigen has been detected in breast (Leivonen et al., 2001), oesophagus (Ikeda et al., 1993), colon (Itzkowitz et al., 1989), pancreas (Kim et al., 2002), stomach (David et al., 1996; Marcos et al., 2011), endometrium (Inoue et al., 1991), and ovary (Numa et al., 1995) carcinomas, whereas low or no expression was observed in the respective normal tissues. STn overexpression was also reported in several cancer precursor lesions, such as esophageal dysplastic squamous epithelia (Itoh et al., 1996), gastric intestinal metaplasia (Baldus et al., 1998; Ferreira et al., 2006) and colonic moderate dysplasia (Cao et al., 1997).

STn is known to influence cell recognition by the immune system (Angata et al., 2007), affect processes as cell cycle, apoptosis, and actin cytoskeleton dynamics, decrease cell–cell aggregation and increase extra-cellular adhesion, migration, invasion (David et al., 1996; Julien et al., 2006, 2005; Pinho et al., 2007) and metastization (Ozaki et al., 2012). In line with these observations, STn positive (STn$^+$) cells have been frequently observed at the invasion front of tumours and in peritoneal and pleural effusions in ovarian cancer patients; yet they are less common in metastatic lesions than in primary tumours (Davidson et al., 2000). In gastric carcinomas, STn was correlated with the depth of invasion and metastization (Ikeda et al., 1993), and thus poor prognosis (Terashima et al., 1998). Conversely, STn was not correlated with the depth of invasion in studies concerning colorectal (Itzkowitz et al., 1989; Ogata et al., 1998) and breast cancers (Schmitt et al., 1995). However, some contradicting results have been presented regarding its association with metastasis and decreased survival in these cancers (Julien et al., 2012). Hence, a recent review suggests that the biological role of STn in tumour development may be dependent on each cancer type or sub-type (Julien et al., 2012).

Despite these observations, there is little information regarding STn in the context of bladder cancer. Given its clinical relevance and the fact that there are available therapies based on this antigen, we addressed the presence of STn in bladder tumours and the mechanisms underlying its expression.

2. Materials and methods

2.1. Patient and sampling

Formalin-fixed, paraffin embedded (FFPE) tissues were prospectively collected from 69 patients, mean age of 69 years (age range 45–89), who underwent transurethral resection (TUR) of the bladder tumour in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011

and May 2012. Based on urothelial carcinoma grading and staging criteria of the World Health Organization (WHO), three different groups were considered (Table 1), low-grade (LG, $n = 24$) and high-grade HG non muscle-invasive (NMIBC, $n = 26$) and muscle-invasive (MIBC, $n = 19$) bladder cancers. Of HG NMIBC, 21 were papillary tumours and 5 were carcinoma *in situ* (CIS). None of these patients had received prior adjuvant therapy. Six normal urothelium tissues of necropsied male individuals without bladder cancer history, within the same mean of age range, were also included.

Additionally, FFPE tissues from 16 radical cystectomy cases including the main lesion in each specimen, responsible for therapeutic decision, the adjacent mucosa, which may or may not include a concomitant tumour, and the ureter representing a distant mucosa, were also studied. Mucosa without visible histopathological alterations was defined as "histologically normal" mucosa.

All procedures were performed under the approval of the Ethics Committee of IPO-Porto, after patient's informed consent. All clinicopathological information was obtained from patients' clinical records.

2.2. Tissue expression of STn and Ki-67

FFPE tissue sections were screened for STn and Ki-67 by immunohistochemistry using the avidin/biotin peroxidase method. Briefly, 3 μm sections were deparaffinised with xylene, rehydrated with graded ethanol series, microwaved for 15 min 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 min. The expression of STn was then evaluated using anti-STn mouse monoclonal antibody, clone TKH2 (Kjeldsen et al., 1988), that identifies both single and clustered STn residues (Ogata et al., 1998), 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 Vectastain Elite ABC peroxidase kit (Vector Lab) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the slides were counterstained with haematoxylin for 1 min. 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^+ tissues were also treated with a neuraminidase from *Clostridium perfringens* (Sigma–Aldrich) as previously described by Marcos et al. (2011) in order to remove the sialic acid. The desialyated samples were thereafter screened for STn. The O-acetylation of Neu5Ac residues in STn was evaluated after treatment with 100 mM NaOH at room temperature for 30 min as described by Ogata et al. (Ogata et al., 1998) prior to immunohistochemistry with antibody TKH2.

A semi-quantitative approach was established to score the immunohistochemical labelling based on the intensity of staining and the percentage of cells that stained positively. 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 proliferative whenever Ki-67 expression was higher than 18%, as described by Santos et al. (Santos et al., 2003).

Table 1 – STn expression in the healthy urothelium and in non-muscle invasive (NMIBC) and muscle invasive (MIBC) bladder cancers of different clinicopathological natures.

	Total	STn expression
Normal urothelium	6	
–		6 (100%)
+		–
++		–
+++		–
Total STn^-		0 (0%)
NMIBC	50	
Low-grade papillary tumours	24	
–		19 (79%)
+		5 (21%)
++		–
+++		–
Total STn^+		5 (21%)
High-grade (CIS + papillary tumours)	26	
Carcinoma in situ (CIS)	5	
–		4 (80%)
+		1 (20%)
++		–
+++		–
Total STn^+		1 (20%)
High-grade papillary tumours	21	
–		5 (24%)
+		9 (43%)
++		4 (19%)
+++		3 (14%)
Total STn^+		16 (76%)
MIBC	19	
–		5 (26%)
+		11 (58%)
++		2 (11%)
+++		1 (5%)
Total STn^+		14 (74%)

–: No reactivity; +: ≤15%; ++: 15–30%; +++: 30–45% of the tumour.

2.3. Cell lines culture

The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and $MCRSTn^+$), were grown as described by Videira et al. (2009b).

2.4. Generation of STn^+ bladder cancer cells

MCR cells were transduced with a retroviral vector generated with the ViraPower™ Lentiviral Expression System (Invitrogen), according to manufacturer's instructions. The whole coding region of human ST6GalNAc.I was PCR amplified and cloned in the pLenti6/V5 Directional TOPO cloning vector which drives the expression of inserted genes through the CMV promoter. A negative control retroviral vector was prepared with an empty plasmid. After transduction with negative control- or ST6GalNAc.I-expressing vectors, MCR cells were selected with 4 μg ml^{-1} blasticidin. An additional immunomagnetic enrichment of the STn^+ cells was performed by using mouse anti-STn (HB-STn1 clone from Dako), followed by the secondary antibody anti-mouse IgG associated to paramagnetic microbeads (Miltenyi Biotec). The stable transduction of the enzyme was confirmed by evaluation of ST6GalNAc.I expression and activity. STn expression was

determined by analysis of the mean fluorescence intensity (MFI) ± SE through flow cytometry analysis using monoclonal antibody TKH2.

2.5. Evaluation of STn expression in cell lines

For phenotypic characterization, cells were stained with 1:50 diluted anti-STn TKH2 monoclonal antibody for 16 h at 4 °C, and 1:100 diluted goat fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Dako) for 15 min at 4 °C in the dark and then acquired in a FacsCalibur Flow cytometer (Becton Dickinson). Data were analysed using the WinMDI v2.9 software (The Scripps Research Institute, San Diego, CA, USA).

2.6. Analysis of ST6GalNAc.I expression

RNA extraction from FFPE sections was performed after deparaffinization of the tissue using Absolutely RNA FFPE kit (Agilent technologies) while for cell lines it was used the GenElute Mammalian Total RNA Purification kit and DNAase treatment (Sigma), according to the manufacturer's instructions. The purity of RNA extracts was determined based on the A_{260}/A_{280} ratio. Only ratios between 1.9 and 2.1 were considered further.

Approximately 250–500 ng of total RNA (1 μg for cell lines) was converted by reverse transcription into cDNA, using the random-primers-based High Capacity cDNA Archive Kit (Applied Biosystems). The expression levels of ST6GalNAc.I were determined by TaqMan assay (Applied Biosystems), the reference sequences detected by each primer/probe set and the Assay ID provided by the manufacturer were the following: ST6GalNAc1 (NM018414.2/Hs00300842_m1). Real time PCR was performed in a 7500 Fast Real-Time PCR System using the TaqMan Universal PCR Master Mix Fast from Applied Biosystems, as described previously by Videira et al. (2007, 2009a). During the cDNA exponential amplification the product formation was proportional to the fluorescence emission resulting from the TaqMan probe degradation (van der Velden et al., 2003). The ST6GalNAc.I mRNA levels were normalized for the expression of β-actin, which was taken as a suitable endogenous control for bladder cancer cells (Videira et al., 2007). The relative mRNA levels were calculated by adapting the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

2.7. Evaluation of ST6GalNAc.I activity

MCR cell pellets were homogenized in H_2O and the protein concentration was determined using the RC-DC protein quantification kit (BioRad) according to the manufacturer's instructions. Sialyltransferase activity was assayed in whole cell homogenates as previously described by Dall'Olio et al. (1997) with some modifications. Briefly, the reaction mixture contained 80 mM sodium cacodylate buffer pH 6.5, 0.5% Triton X-100, 6 μg μl^{-1} of asialo bovine submaxillary mucin (ABSM, prepared by acid desialylation of BSM) as acceptor substrate, 30 μM (1280 Bq) of CMP-[^{14}C]Sia (Amersham) and 2 μg μl^{-1} of homogenate proteins. Endogenous controls were prepared in the absence of acceptor substrate. The enzyme reactions were incubated at 37 °C for 2 h and the acid insoluble radioactivity was measured as previously described by Dall'Olio et al. (1997). The incorporation on endogenous substrates was subtracted.

2.8. Cell proliferation measurement

To study their proliferative capacity, cells were labelled with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). The MCR cells were resuspended into medium at final concentration of 1×10^6 cells ml^{-1} and incubated with 10 μM CFSE, following the manufacturer's instructions. Subsequently, the CFSE-labelled cells were seeded into 24-well microplates, incubated in a 5% CO_2 incubator at 37 °C and harvested at 24, 48, 72 and 96 h post-culture. Flow cytometry using a FACSCalibur (Becton–Dickinson) was performed and the data collected were analysed with ModFit LT 3.2 software (Verity Software House, Topsham, ME), allowing to assess the cell proliferation index (PI). The PI represents the average number of cells that were originated from a single cell of the parental generation. The parental generation was set based on the analysis of data obtained from the cells corresponding to the 24 h of culture.

2.9. Analysis of cell motility using a wound-healing assay

Cell motility was tested in a wound-healing migration assay. MCR cells were seed into 12-well microplates and grown to confluency. A scratch was made in the monolayer with a sterile 200 μl pipette tip. After wounding, the suspended cells and debris were washed away and fresh medium was added. At 0 and 24 h after wounding, scratched regions were photographed with an inverted microscope equipped with a digital camera.

2.10. Invasion assay

Invasion assays were performed using BD Biocoat Matrigel™ invasion chambers, comprised by an 8-μm diameter pore size filter coated with a thin layer of matrigel, and placed in a two-compartment system in a 24-well plate. Prior to each experiment, filters were re-hydrated in serum-free DMEM medium for 2 h at 37 °C. After detachment of subconfluent cells with trypsin/EDTA, cells were suspended in culture medium supplemented with 5% inactivated FBS, counted and seeded on the upper side of the matrigel-coated filter at a density of 5×10^4 cells/well. After 24 h at 37 °C, filters were fixed in 4% paraformaldehyde and non-invading cells, present on the upper side, were completely removed, to facilitate analysis. Cells that had invaded the underside of the filters were mounted in Vectashield+4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA), and visualized through a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Germany). Invasive cells were scored in at least 12 microscopic fields (20× objective) when DAPI-counterstained nuclei passed through the filter pores. Results are presented as means ± SD for each sample. Invasion levels are expressed as a ratio of the results obtained with the mock-transfected control cell line.

2.11. Statistical analysis

Statistical analysis was performed using the Student's *T*-test for unpaired samples. Differences were considered to be significant when $p < 0.05$. A chi-square test was used to analyse correlations between clinicopathological features and STn and Ki-67 expressions.

3. Results

3.1. Expression of STn in bladder tumours

STn expression in bladder tumours was evaluated by immunohistochemistry using mouse monoclonal antibody clone TKH2. As shown in Table 1, STn is not expressed in the healthy urothelium; conversely 46% of the bladder tumours presented cells with STn membrane and cytoplasmic staining (32/69) (Figure 1), demonstrating the tumour-specific nature of this antigen. The removal of sialic acids from the tissue sections with a α-neuraminidase impaired the recognition by TKH2 and confirmed STn expression.

STn expression was lower in low-grade (LG) NMIBC (21% STn^+ tumours; Figure 1A–B) compared to high-grade lesions (HG; 67%), which include papillary tumours (76% STn^+ tumours; Figure 1C–E), CIS (20% of STn^+ tumours; Figure 1F), and MIBC (74% STn^+ tumours; Figure 1G–H). Noteworthy, STn was absent from the majority of CIS (4/5; 80%) and showed an expression comparable to LG tumours. Altogether, these results highlight an association between the STn antigen and high grade NMIBC ($p < 0.002$; Figure 2) as well as with muscle invasive tumours ($p < 0.03$; Figure 2).

The O-acetylation of sialic acid residues prevents TKH2 from recognizing STn antigens in certain tissues (Ogata et al., 1998). To exclude this possibility in bladder cancer, the slides were chemically de-O-acetylated prior to immunohistochemistry. This procedure did not alter STn expression patterns demonstrating that STn antigens were not encrypted by O-acetylation.

3.1.1. Pattern and extension of STn expression in bladder tumours

The STn antigen presented a focal expression that for the majority of the STn positive cases (26/36) did not exceeded 15% of the tumour section (Table 1). Furthermore, in 25% of the STn positive cases (9/36) the antigen was detected in less than 5% of the tissue (data omitted from Table 1). Higher expression patterns were restricted to HG papillary NMIBC, where 27% of the cases (7/26) presented STn levels between 15% and 45% of the tumour section (Table 1) and locally diffuse staining (Figure 1C, D, G). STn was mainly observed in basal layer cells (75% of STn^- cases; Figure 1A, C–E), but it could be also detected throughout the papillae (Figure 1C–E) and cells of the luminal surface (Figure 1F) in cases presenting locally diffuse staining. STn was further observed in cells invading the basal (50% of STn^+ of HG NMIBC; Figure 1C–E, G) and muscle layers (57% of STn^+ MIBC; Figure 1G, H), suggesting a role in invasion.

3.1.2. STn antigen expression in advanced tumours and in the surrounding areas

The STn antigen was also evaluated in a series of radical cystectomy specimens which included the tumour used for therapeutic decision (termed "main tumour" in Figure 3) and the tumour-adjacent mucosa. The ureters were included as distant mucosa (Figure 3). In agreement with the observations from Table 1, STn was detected in 69% (11/16) of all main tumours as well as in their adjacent mucosa (Figure 3), independently of their histological classification. Noteworthy, STn was absent from 90% of the distant mucosas of STn positive cases; the only exceptions being a ureter with pre-neoplastic and another with a neoplastic lesions (Figure 3). These results point out that the STn^+ tumour-adjacent mucosa may display molecular changes similar to those of the main lesions. Thus, this antigen may be useful as a marker of field carcinogenesis in the bladder.

3.2. Expression of ST6GalNAc.I in bladder tumours

The presence of STn has been strongly associated with the overexpression of ST6GalNAc.I in several human malignancies. To assess this event in bladder tumours, mRNA levels of *ST6GalNAc.I* gene were analysed and normalized in relation to β-actin, which proved to be a stable expressed gene in previous studies concerning bladder tumours (Videira et al., 2007). As shown by Figure 4, low gene expression levels were detected in tumours that did not express STn. In addition, the levels of *ST6GalNAc.I* increased with the expression of STn, and were significantly higher in the tumours with STn expression superior to 15%. Figure 4 also shows that this behaviour was similar in LG and HG tumours. However, as a result of higher STn expression, the average ST6GalNAc.I mRNA levels were more elevated in HG (53%) tumours than LG (9%). These observations suggest that overexpression of *ST6GalNAc.I* gene is one of the main events leading to STn expression in bladder tumours.

3.3. STn expression and tumour proliferation

As shown above, the expression pattern of STn correlates with HG tumours, known to present elevated proliferation rates (Margulis et al., 2009; Santos et al., 2003). To assess a possible association between STn and proliferation, 24 cases from the initial series of 69 bladder tumours, comprehending 12 LG and 12 HG tumours (7 NMIBC, none of them CIS, and 5 MIBC), were screened for STn and Ki-67 expression. Tumours presenting Ki-67 expression superior to 18% were classified as proliferative. As highlighted by the graphical matrix in Figure 5A, 8% (1/12) LG and 75% (9/12) HG cases showed elevated Ki-67, confirming the higher proliferation of HG tumours ($p < 0.0012$). Similarly, Figure 5A also shows an association between proliferative phenotypes and STn expression ($p < 0.001$). However, in all STn positive cases, the examination of sequential sections revealed that STn antigen expression was mainly seen in areas that did not express Ki-67 (Figure 5A), although some overlap was present in 25% of the cases (3/12; Figure 5B). This indicates that the STn antigen is mostly expressed in non-proliferative areas of the tumour. Nevertheless, the majority of the non-proliferative tumours also did not express STn (12/14), demonstrating an interdependence between both phenomena.

3.4. In vitro assessment of the biological significance of STn expression

3.4.1. Development of a high-grade bladder cancer cell line overexpressing STn

To further corroborate the role of ST6GalNAc.I in the expression of STn antigen by bladder cancer cells, we induced the overexpression of ST6GalNAc.I in a bladder cancer cell line. The MCR bladder cell line, that showed negligible expression

of ST6GalNAc.I and no STn (data not shown), was transduced with a lentivirus expressing the coding region of the human *ST6GalNAcI* gene. The obtained cell line variant, herein named MCRSTn$^+$, showed markedly increased expression of *ST6GalNAcI* mRNA levels (Figure 6A). It also showed significantly higher sialyltransferase activity towards the ABSM, a substrate for the ST6GalNac.I enzyme, when compared with the negative control cell line (MCRnc) transduced with void lentivirus (Figure 6A). The overexpression of STn antigen by MCRSTn$^+$ cell line variant was confirmed by flow cytometric analysis (Figure 6B).

3.4.2. *STn influence on cell proliferation, migration and invasion*

STn expression was correlated with tumours with higher proliferative indexes (Figure 5). To assess the influence of STn in

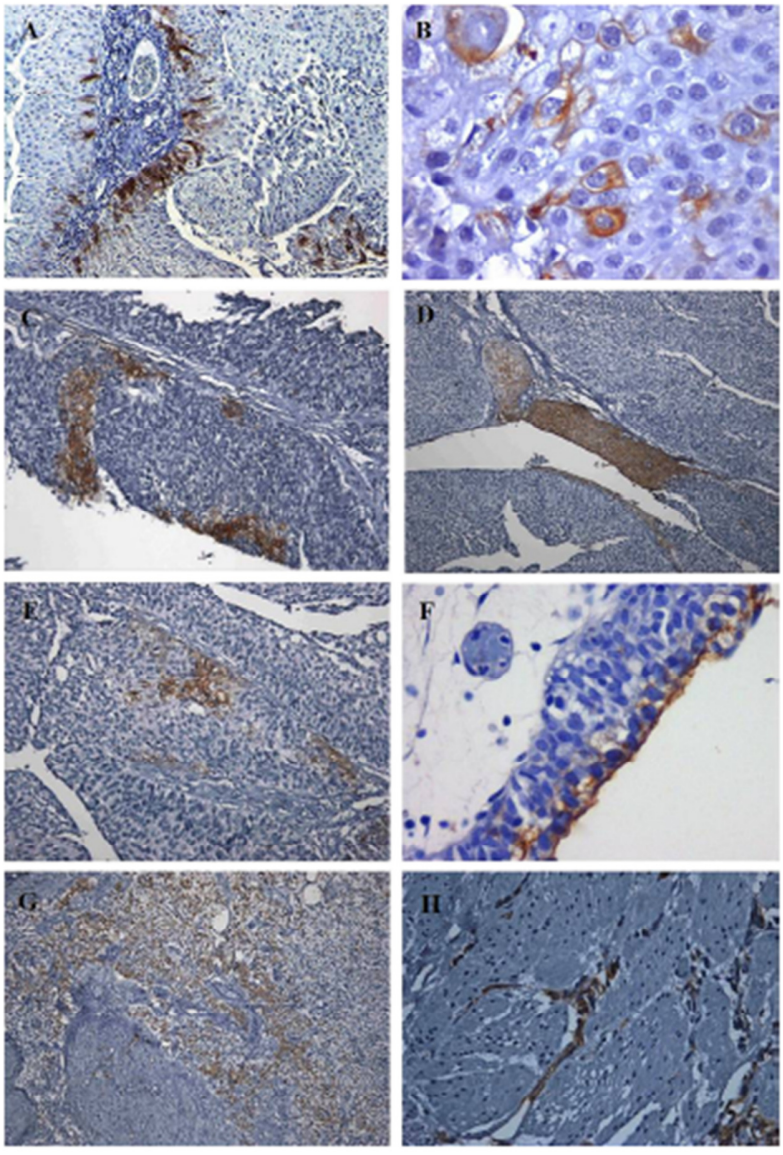

Figure 1 – Expression of STn in FFPE bladder tumours. A) Low-grade papillary tumour showing a predominance of STn$^+$ cells in the basal layer; B) Magnification which shows tumour cells with membrane and cytoplasmic STn$^+$ staining; C) High-grade papillary tumour evidencing the focal nature of STn expression. Positive cells were found both in the basal layer and throughout the papillae; D) High-grade papillary tumour showing locally extensive STn positivity; E) High-grade papillary tumour evidencing STn$^+$ in the basal layer; F) CIS showing STn$^+$ in the cells facing the lumen of the bladder; G) MIBC showing locally extensive STn expression including at the muscle invasive front; H) MIBC highlighting STn$^+$ cells invading the muscle layer.

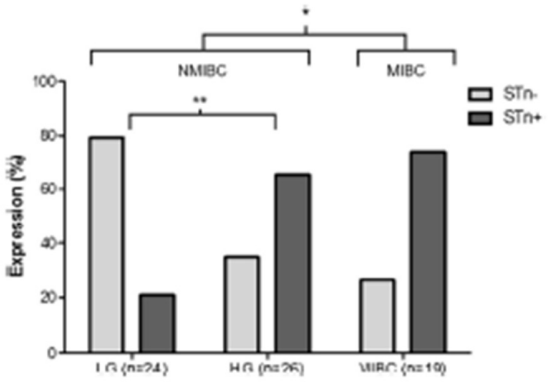

Figure 2 – Association between STn expression and HG NMIBC and MIBC. The percentage of STn$^+$ tumours was higher in HG when compared to LG and also in MIBC when compared to NMIBC (LG + HG). "*" $p = 0.03$; "" $p = 0.002$ (Chi-square test).**

proliferation, MCR cells (MCRnc and MCRSTn$^+$) were cultured for 48, 72 and 96 h and then evaluated in relation to their proliferation index. The comparison between the two cell line variants showed that the proliferation index of MCRSTn$^+$ cells was generally higher than the index of MCRnc cells, although only statistically different at 72 h of culture ($p < 0.05$; Figure 7). However, this effect was no longer significant at 96 h of culture (Figure 7).

STn positive cells were observed invading the basal and muscle layers (Figures 1 and 2) and in the adjacent mucosa of advanced stage bladder tumours (Figure 5), suggesting a correlation of STn with invasion and migration. Thus, the influence of STn expression in MCR cell invasion was assessed using the Matrigel invasion assay. Our results evidence that

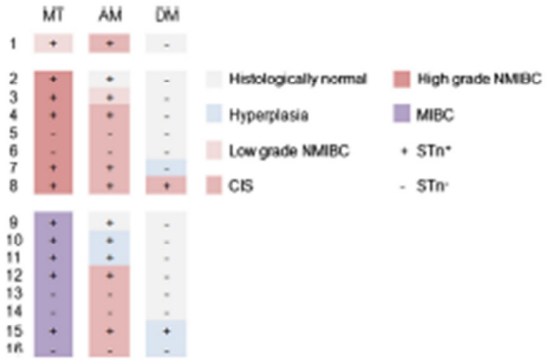

Figure 3 – Expression pattern of STn in radical cystectomy specimens. Radical cystectomy specimens have been organized based on histological grade. They include the tumour responsible by the therapeutic decision termed "main tumour" (MT), an adjacent (AM) and distant mucosa (DM). The graphical matrix highlights that, whenever STn is expressed by the main tumour (13/16; 63%), it is always present in the adjacent mucosa (13/13, 100%). One preneoplastic and one neoplastic distant mucosa also expressed the antigen.

MCR cells transduced with ST6GalNAc.I (MCRSTn$^+$) are approximately four folds more invasive than bladder cells transduced with the negative control (MCRnc; Figure 8A). The effect of STn expression on cell migration was estimated by a wound-healing assay. Therefore, uniform scratches were made in confluent monolayers of MCRnc and MCRSTn$^+$ cell lines and the capability of the cells to migrate and fill the scratches was monitored. As observed in Figure 8A, by 24 h after wounding, the MCRSTn$^+$ cells had almost completely covered the empty space. Conversely, the negative control, MCRnc cells, displayed a large "gap", thus demonstrating their lower capability to closure the wound. Our results evidence that MCR cells expressing STn present increased invasion and wound repair capacities.

4. Discussion

The STn antigen is highly expressed by several human carcinomas and preneoplastic lesions (Julien et al., 2012) and is explored as a tumour marker in serological assays (CA72-4) (Reis et al., 2010).

Despite the clinical relevance of STn in human malignancies, scarce information is available about its role in bladder tumours. Over twenty years ago, Langkilde et al. (1992) addressed this antigen on series of transitional cell carcinomas (currently classified as high-grade urothelial cell carcinomas according to current WHO guidelines (Babjuk et al., 2012)). Normal mucosal biopsy specimens from patients with non-malignant bladder urologic diseases were included as controls. According to the authors, STn was not expressed by the control group, showed a very restricted pattern of expression in bladder tumours and no association with recurrence and progression. Subsequent *in vitro* studies found that

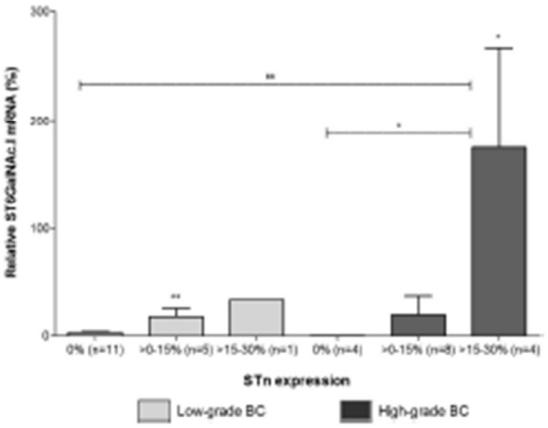

Figure 4 – Association between ST6GalNAc.I and STn expression in LG and HG bladder tumours. The graph shows that ST6GalNAc.I expression is increased in STn$^+$ tumours and increases further for more elevated STn expressions (> 15–30% of the tumour section). This suggests that the overexpression of ST6GalNAc.I is one of the main mechanisms underlying the presence of STn in bladder cancers. Furthermore, it shows this event occurs in both LG and HG tumours. "*" $p < 0.05$; "" $p < 0.01$ (Student's *T*-test).**

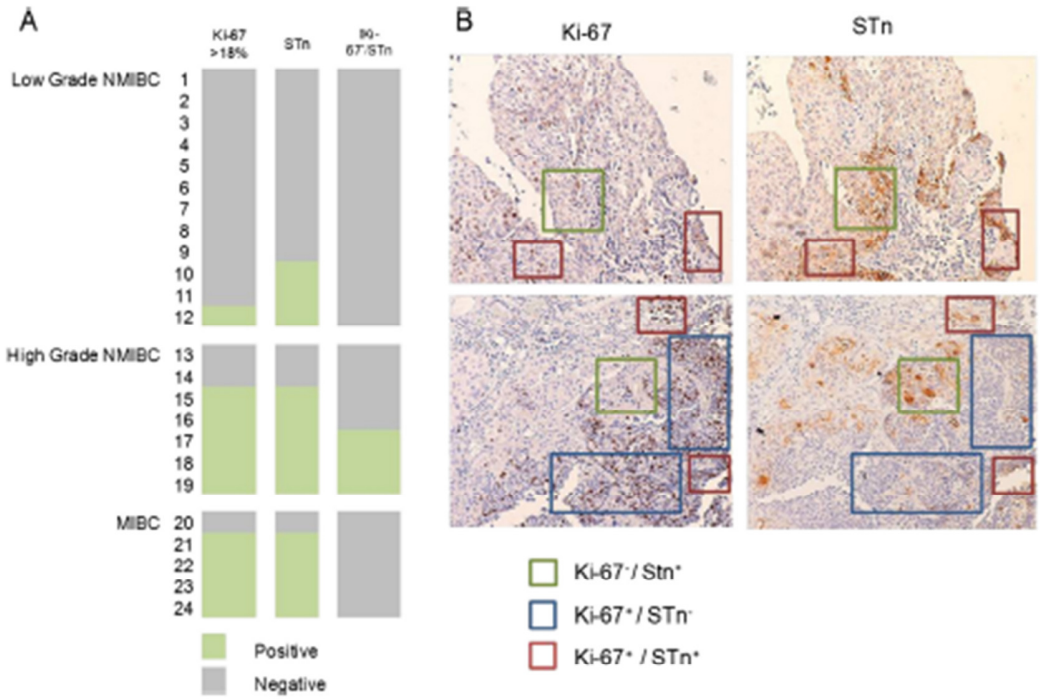

Figure 5 – Expression of STn and Ki-67 in bladder tumours. A) graphical matrix highlighting the association between proliferative tumours (Ki-67 > 18%) and STn expression in bladder tumours. HG NMIBC and MIBC were considered to be proliferative tumours and a significant association was found between STn expression and tumours presenting proliferation phenotypes ($p < 0.001$; Chi-square test). The notation "Ki-67/STn⁺" in the column more to the right refers to tumours presenting areas that appear to exhibit cells expressing both Ki-67 and STn. B) Immunohistochemistry for Ki-67 and STn highlighting Ki-67⁻/STn⁺; Ki-67⁺/STn⁻; and Ki-67⁺/STn⁺ areas.

mucins MUC1, MUC2 and MAUB (mucin antigen of the urinary bladder) isolated from bladder cancer cell lines carried STn (Bergeron et al., 1996, 1997). However, no evidence of such an expression was found in tumours. Herein, we readdressed this matter and found that the STn antigen was associated with advanced stage bladder tumours. More important, STn was absent in the healthy urothelium, which demonstrates its tumour-associated nature. Since this study was performed on a recent prospective series it is not possible, at this point, to determine correlations with disease outcome. Nevertheless, STn was mainly expressed by HG papillary NMIBC, known for their elevated risk of recurrence and progression to muscle invasive disease and MIBC that encompass an elevated risk of metastization and present decreased overall survival (Babjuk et al., 2012). STn expression was further associated with elevated Ki-67, a proliferation-related molecule and a surrogate biomarker of increased risk to recurrence and progression in bladder tumours (Margulis et al., 2009; Santos et al., 2003). In addition, the majority of non-proliferative tumours did not express STn, which demonstrates that the expression of the antigen is indeed a characteristic of proliferative tumours. Still, STn was mainly detected in non-proliferative areas of the tumours. However, the STn antigen was frequently observed in areas of invasion of the basal and muscle layers, suggesting it may be associated with the process of cell migration and invasion. This reinforces the notion that STn is part of a malignant bladder cancer phenotype, as previously observed for other carcinomas (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007). We also found the STn antigen in tumour-adjacent mucosa, which may be explained by the migration of STn⁺ cells to the tumour surroundings. On the other hand, this may be a consequence of field carcinogenesis previously observed in bladder cancers (Jones et al., 2005; Palmeira et al., 2011). Nevertheless, the STn antigen holds potential as a biomarker of bladder disseminated disease.

STn is a product of an incomplete O-glycosylation process due to the premature O-6 sialylation of the glycoside GalNAcα1-O-Ser/Thr (Tn antigen) by ST6GalNAc.I (Marcos et al., 2004). In several epithelial tumours STn results from an increased ST6GalNAc.I expression and/or activity (Marcos et al., 2011; Sewell et al., 2006; Vazquez-Martin et al., 2004). Previous studies have reported ST6GalNAc.I expression by the urothelium at the mRNA level (Yamamoto et al., 2003); however we and others (Langkilde et al., 1992) have not detected STn expression in the histologically healthy tissues. These observations suggest either the absence of the antigen or the insufficient sensitivity of the method. ST6GalNAc.I localization in the Golgi apparatus and the competitive action of other glycosyltransferases for the Tn antigen may also favour the extension of

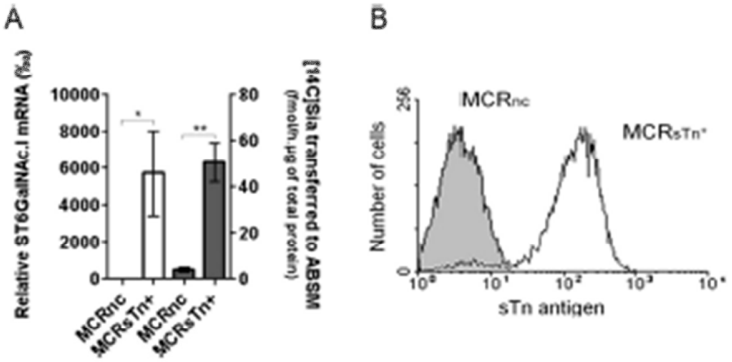

Figure 6 – ST6GalNAc.I mRNA expression and sialyltransferase activity in bladder cancer MCR cell lines. A) ST6GalNAc.I expression and activity in MCR cell lines. The relative mRNA levels of ST6GalNAc.I (open bars) and sialyltransferase activity towards ABSM (grey bars) were analysed as described in the Material and methods section. Both, ST6GalNAc.I mRNA and sialyltransferase activity towards ABSM are negligible in negative control cells and markedly increased upon ST6GalNAc.I transduction. B) Flow cytometry analyses of transduced MCR cells. Both negative control (MCRnc in grey histogram) and ST6GalNAc.I-transduced (MCRSTn$^+$ in open histogram) cell lines were stained with the secondary antibody anti-Ig's-FITC following incubation with the primary antibody anti-STn antigen. 90% of the ST6GalNAc.I-transduced cells expressed the STn antigen (MFI = 216). The data are shown as a mean ± standard deviation of 3 independent studies. "*" $p < 0.05$, "" $p < 0.01$ (Student's *T*-test).**

the O-glycan chain in non-pathological conditions. On the other hand we showed that the levels of STn in bladder tumours were correlated with the expression of ST6GalNAc.I, supporting this as a major molecular mechanism underlying STn biosynthesis in these tumours. Few cases presented STn expression associated with a basal level of ST6GalNAC.I, meaning that other factors may contribute to promote the biosynthesis of STn. A disorganization of secretory organelles (Sewell et al., 2006), somatic mutations in the gene *Cosmc*, encoding a molecular chaperone essential for O-chain elongation (Ju et al., 2008), the down-regulation/decreased activity of several other glycosyltransferases and/or the availability of sugar donors for biosynthesis, may also lead to STn overexpression. The integrated study of metabolic pathways, glycosyltransferases expression/activity, intra-cellular ultrastructures and microenvironmental changes may further enlighten the molecular events leading to abnormal O-glycosylation of bladder cancer proteins.

In addition we have screened HT1376, 5637, T24 and MCR bladder cancer cell lines and found neglectable levels of the STn antigen (data not shown). The same was previously observed in gastric (Ozaki et al., 2012; Pinho et al., 2007) and breast (Clement et al., 2004; Julien et al., 2005, 2006) cancers cell models, demonstrating that tumour cells may lose the ability to express this antigen *in vitro*. Microenvironmental factors may play a determinant role in the induction of STn biosynthesis, yet these events remain unknown. Following the association of STn with invasive cases, we elected the invasive bladder cancer cell line MCR to evaluate the biological role of STn in these tumours. We started by stably transducing the MCR cells with ST6GalNAc.I, which resulted in the overexpression of STn. The expression of STn did not promote a significant enhancement of MCR cell proliferation, which is agreement with observations made for breast (Clement et al., 2004; Julien et al., 2005, 2006) and gastric cancer models (Pinho et al., 2007). These findings associated with the absence of the antigen from most bladder tumours non-proliferative areas strongly suggests that STn expression does not play a direct role in tumour proliferation.

On the other hand, STn expression significantly enhanced the migration and invasive capacity of MCR cells, demonstrating that this antigen plays an important role in bladder cancer cell invasion, as suggested by the observation of bladder tumours. Enhanced migration capabilities of STn$^+$ cells on components of the extracellular matrix, such as fibronectin and collagen, have been described for other cancer cell lines (Julien et al., 2005, 2006; Pinho et al., 2007), and result, among several factors, from impaired integrin binding (Clement et al., 2004). In addition, STn expression has been shown to increase the invasion potential of tumour cells (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007), supporting a similar role in bladder tumours. Further experiments are however required to clarify the molecular mechanisms underlying promotion of cancer cell invasion and migration. These findings reinforce however that alterations in the glycosylation patterns of cell-surface proteins may strongly interfere with events like cell–cell adhesion, cell–matrix interaction, tumour growth, motility and invasion (Dall'Olio et al., 2012).

In resume, our work comprehensively describes the expression of the STn antigen in bladder cancer. Namely, it demonstrates the tumour-specific nature of this type of glycosylation and its association with advanced, highly proliferative tumours, invasion and organ disseminated disease. Thus, the evaluation of STn antigen may add valuable information about the aggressiveness of proliferative tumours, complementing the information given by Ki-67. Studies are ongoing in broader retrospective series to determine the association of STn with disease outcome and corroborate these findings. We are also devoted to the identification of the glycoproteins yielding STn, which is expected to bring insights

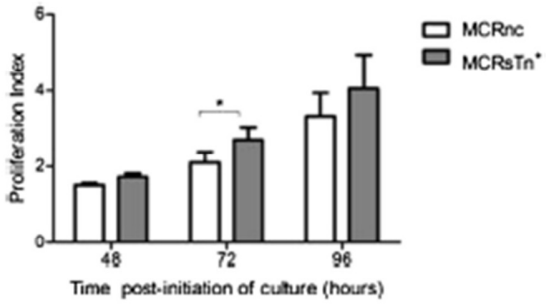

Figure 7 – Comparison between the proliferation capacity of MCRnc and MCRSTn$^+$ cells. The transduced MCR cells were labelled with CFSE and cultured for various periods of time (48, 72 and 96 h). The cells were harvested and analysed by flow cytometry with Modfit software, allowing the calculation of the proliferation index, which represents the average number of cells that was originated by a single cell of the parent generation. At the various periods of culture, MCRSTn$^+$ cells show a higher proliferation index than the negative control, but this difference was only statistically significant at 72 h of culture. The data are presented as a mean ± standard deviation of 3 independent studies. "*" $p < 0.05$ (Student's *T*-test).

about the role of this type of glycosylation in bladder carcinogenesis and provide novel therapeutic vectors. The antigen STn may also be monitored noninvasively in urine or serum using as is the case for other human carcinomas using the CA72-4 test (Reis et al., 2010). This could allow decreasing the number of cystectomies in post-surgery follow-ups of patients with high-grade tumours, a particularly critical matter for the elderly that constitute the majority of the cases.

Furthermore, the STn antigen is associated to high-grade NMIBC which currently constitutes one of the main therapeutics concerns due to their elevated risk of recurrence/progression (Babjuk et al., 2012). Adjuvant immunotherapy with BCG has allowed to delay recurrence and decrease the risk of progression into muscle invasive disease (Babjuk et al., 2012); still more than half of the patients either recur within two-years after TUR of the tumour or show intolerance to the treatment (Askeland et al., 2012; Yates and Roupret, 2011). Due to the lack of efficient therapies, upon therapeutic failure and/or muscle invasion, the patient is faced with cystectomy (Babjuk et al., 2012).

Carbohydrate antigens associated with advanced-stage tumours and malignant phenotypes such as STn, are expressed at the cell surface and, therefore, available for antibody or lectin-mediated recognition (Neutsch et al., 2012). Thus, these antigens may present an opportunity for the introduction of novel therapeutics, such as selective drug-delivery approaches (Neutsch et al., 2012) or carbohydrate-based immunotherapy (Heimburg-Molinaro et al., 2011). An anti-cancer vaccine named Theratope, comprehending a synthetic STn coupled to the immunogenic carrier keyhole limpet haemocyanin has already been developed (Julien et al., 2009; Miles et al., 2011; Sandmaier et al., 1999). Tests in animal models and humans for breast, ovarian, and colorectal cancers have showed that the antigen is safe and produces a strong immune response against these tumours (Julien et al., 2009, 2012; Miles et al., 2011). Even though Theratope failed to improve overall survival of metastatic breast cancer patients in a phase III clinical study, the design of the study disregarded the heterogeneous STn expression between patients (Miles et al., 2011), compromising the outcome (Julien et al., 2012; Zeichner, 2012). Thus, Theratope or other STn-based vaccine designs may constitute valuable therapeutic options for STn positive advanced bladder tumours. However, given the low association of STn with more proliferative areas of the tumour, one is led to speculate that advanced stage bladder cancer patients may better benefit from the combination of anti-STn immunotherapy and anti-proliferative drugs. Furthermore, these approaches may allow targeting disseminated disease in the adjacent and distant mucosa from the main tumour.

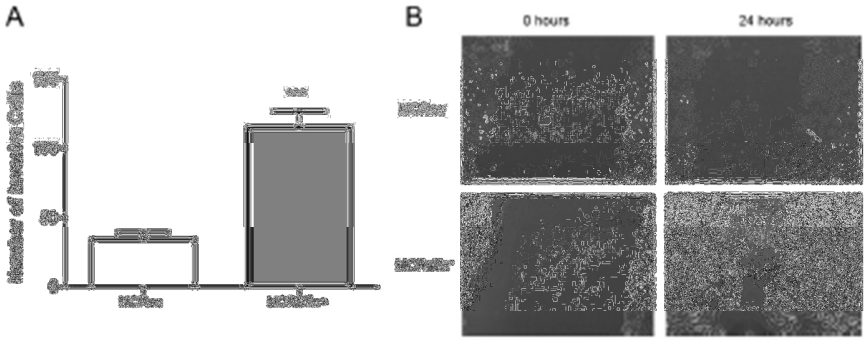

Figure 8 – STn expression promotes MCR cells wound healing closure and invasion. A) Wound healing closure assay. Uniform scratches were made using a 200 μL pipette tip in confluent monolayers of MCRSTn$^+$ and MCRnc cells. Cells were allowed to heal and the extent of closure was monitored by microscopic analysis. After 24 h culture, the MCRSTn$^+$ cells had almost completely covered the wound, in clear contrast to negative control, MCRnc, where unoccupied space was still observed. B) Invasion assay. MCRSTn$^+$ and MCRnc cells were incubated for 24 h, in the upper compartment of Matrigel invasion chambers, in complete DMEM medium and in the absence of other chemoattractants. Invasive cells were determined as described in Materials and methods. The data are presented as a mean ± standard deviation of 4 independent studies. "*" $p < 0.001$ (Student's *T*-test).

Acknowledgements

This work was supported by Portuguese Foundation for Science and Technology (FCT) Postdoctoral grant SFRH/BPD/66288/2009 (José Alexandre Ferreira), PhD grant SFRH/BD/43399/2008 (Luis Lima), SFRH/BD/81860/2011 (Mariana Silva), SFRH/BD/45120/2008 (Paulo F. Severino) and by LPPC/Pfizer 2011 (Mylene Carrascal). This work was also financially supported by FCT (PTDC-SAU-ONC/112511/2009). FCT is co-financed by European Social Fund (ESF) under Human Potential Operation Programme (POPH) from National Strategic Reference Framework (NSRF).

REFERENCES

Angata, T., Tabuchi, Y., Nakamura, K., Nakamura, M., 2007. Siglec-15: an immune system Siglec conserved throughout vertebrate evolution. Glycobiology 17, 838–846.

Askeland, E.J., Newton, M.R., O'Donnell, M.A., Luo, Y., 2012. Bladder cancer immunotherapy: BCG and beyond. Adv. Urol. 2012, 181987.

Babjuk, M., Oosterlinck, W., Sylvester, R., Kaasinen, E., Bohle, A., Palou-Redorta, J., Roupret, M., 2012. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. Actas Urol. Esp. 36, 389–402.

Baldus, S.E., Zirbes, T.K., Monig, S.P., Engel, S., Monaca, E., Rafiqpoor, K., Hanisch, F.G., Hanski, C., Thiele, J., Pichlmaier, H., Dienes, H.P., 1998. Histopathological subtypes and prognosis of gastric cancer are correlated with the expression of mucin-associated sialylated antigens: Sialosyl-Lewis(a), Sialosyl-Lewis(x) and sialosyl-Tn. Tumour. Biol. 19, 445–453.

Bergeron, A., Champetier, S., LaRue, H., Fradet, Y., 1996. MAUB is a new mucin antigen associated with bladder cancer. J. Biol. Chem. 271, 6933–6940.

Bergeron, A., LaRue, H., Fradet, Y., 1997. Biochemical analysis of a bladder-cancer-associated mucin: structural features and epitope characterization. Biochem. J. 321 (3), 889–895.

Cao, Y., Schlag, P.M., Karsten, U., 1997. Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycotopes (TF, Tn, and sialosyl-Tn) in benign and malignant lesions of colonic epithelium: apolar localization corresponds to malignant transformation. Virchows Arch. 431, 159–166.

Clement, M., Rocher, J., Loirand, G., Le, P.J., 2004. Expression of sialyl-Tn epitopes on beta1 integrin alters epithelial cell phenotype, proliferation and haptotaxis. J. Cell Sci. 117, 5059–5069.

Conze, T., Carvalho, A.S., Landegren, U., Almeida, R., Reis, C.A., David, L., Soderberg, O., 2010. MUC2 mucin is a major carrier of the cancer-associated sialyl-Tn antigen in intestinal metaplasia and gastric carcinomas. Glycobiology 20, 199–206.

Dall'Olio, F., Malagolini, N., Trinchera, M., Chiricolo, M., 2012. Mechanisms of cancer-associated glycosylation changes. Front. Biosci. 17, 670–699.

Dall'Olio, F., Mariani, E., Tarozzi, A., Meneghetti, A., Chiricolo, M., Lau, J.T., Facchini, A., 1997. Expression of beta-galactoside alpha 2,6-sialyltransferase does not alter the susceptibility of human colon cancer cells to NK-mediated cell lysis. Glycobiology 7, 507–513.

David, L., Carneiro, F., Sobrinho-Simoes, M., 1996. Sialosyl Tn antigen expression is associated with the prognosis of patients with advanced gastric cancer. Cancer 78, 177–178.

Davidson, B., Berner, A., Nesland, J.M., Risberg, B., Kristensen, G.B., Trope, C.G., Bryne, M., 2000. Carbohydrate antigen expression in primary tumors, metastatic lesions, and serous effusions from patients diagnosed with epithelial ovarian carcinoma: evidence of up-regulated Tn and Sialyl Tn antigen expression in effusions. Hum. Pathol. 31, 1081–1087.

Dovedi, S.J., Davies, B.R., 2009. Emerging targeted therapies for bladder cancer: a disease waiting for a drug. Cancer Metastasis Rev. 28, 355–367.

Ferreira, B., Marcos, N.T., David, L., Nakayama, J., Reis, C.A., 2006. Terminal alpha1,4-linked N-acetylglucosamine in Helicobacter pylori-associated intestinal metaplasia of the human stomach and gastric carcinoma cell lines. J. Histochem. Cytochem. 54, 585–591.

Gilewski, T.A., Ragupathi, G., Dickler, M., Powell, S., Bhuta, S., Panageas, K., Koganty, R.R., Chin-Eng, J., Hudis, C., Norton, L., Houghton, A.N., Livingston, P.O., 2007. Immunization of high-risk breast cancer patients with clustered sTn-KLH conjugate plus the immunologic adjuvant QS-21. Clin. Cancer Res. 13, 2977–2985.

Hakomori, S., 2001. Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv. Exp. Med. Biol. 491, 369–402.

Heimburg-Molinaro, J., Lum, M., Vijay, G., Jain, M., Almogren, A., Rittenhouse-Olson, K., 2011. Cancer vaccines and carbohydrate epitopes. Vaccine 29, 8802–8826.

Hussain, M.H., Wood, D.P., Bajorin, D.F., Bochner, B.H., Dreicer, R., Lamm, D.L., O'Donnell, M.A., Siefker-Radtke, A.O., Theodorescu, D., Dinney, C.P., 2009. Bladder cancer: narrowing the gap between evidence and practice. J. Clin. Oncol. 27, 5680–5684.

Ikeda, Y., Kuwano, H., Baba, K., Ikebe, M., Matushima, T., Adachi, Y., Mori, M., Sugimachi, K., 1993. Expression of sialyl-Tn antigens in normal squamous epithelium, dysplasia, and squamous cell carcinoma in the esophagus. Cancer Res. 53, 1706–1708.

Inoue, M., Ogawa, H., Tanizawa, O., Kobayashi, Y., Tsujimoto, M., Tsujimura, T., 1991. Immunodetection of sialyl-Tn antigen in normal, hyperplastic and cancerous tissues of the uterine endometrium. Virchows Arch. A Pathol. Anat. Histopathol. 418, 157–162.

Itoh, T., Yonezawa, S., Nomoto, M., Ueno, K., Kim, Y.S., Sato, E., 1996. Expression of mucin antigens and Lewis X-related antigens in carcinomas and dysplasia of the pharynx and larynx. Pathol. Int. 46, 646–655.

Itzkowitz, S.H., Yuan, M., Montgomery, C.K., Kjeldsen, T., Takahashi, H.K., Bigbee, W.L., Kim, Y.S., 1989. Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. Cancer Res. 49, 197–204.

Jones, T.D., Wang, M., Eble, J.N., MacLennan, G.T., Lopez-Beltran, A., Zhang, S., Cocco, A., Cheng, L., 2005. Molecular evidence supporting field effect in urothelial carcinogenesis. Clin. Cancer Res. 11, 6512–6519.

Ju, T., Lanneau, G.S., Gautam, T., Wang, Y., Xia, B., Stowell, S.R., Willard, M.T., Wang, W., Xia, J.Y., Zuna, R.E., Laszik, Z., Benbrook, D.M., Hanigan, M.H., Cummings, R.D., 2008. Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. Cancer Res. 68, 1636–1646.

Julien, S., Adriaenssens, E., Ottenberg, K., Furlan, A., Courtand, G., Vercoutter-Edouart, A.S., Hanisch, F.G., Delannoy, P., Le, B.X., 2006. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology 16, 54–64.

Julien, S., Lagadec, C., Krzewinski-Recchi, M.A., Courtand, G., Le, B.X., Delannoy, P., 2005. Stable expression of sialyl-Tn antigen in T47-D cells induces a decrease of cell adhesion and an increase of cell migration. Breast Cancer Res. Treat. 90, 77–84.

Julien, S., Picco, G., Sewell, R., Vercoutter-Edouart, A.S., Tarp, M., Miles, D., Clausen, H., Taylor-Papadimitriou, J., Burchell, J.M., 2009. Sialyl-Tn vaccine induces antibody-mediated tumour

protection in a relevant murine model. Br. J. Cancer 100, 1746–1754.
Julien, S., Videira, P.A., Delannoy, P., 2012. Sialyl-Tn in cancer: (how) did we miss the target? Biomolecules 2, 435–466.
Kim, G.E., Bae, H.I., Park, H.U., Kuan, S.F., Crawley, S.C., Ho, J.J., Kim, Y.S., 2002. Aberrant expression of MUC5AC and MUC6 gastric mucins and sialyl Tn antigen in intraepithelial neoplasms of the pancreas. Gastroenterology 123, 1052–1060.
Kjeldsen, T., Clausen, H., Hirohashi, S., Ogawa, T., Iijima, H., Hakomori, S., 1988. Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2-6 alpha-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res. 48, 2214–2220.
Lakshminarayanan, V., Thompson, P., Wolfert, M.A., Buskas, T., Bradley, J.M., Pathangey, L.B., Madsen, C.S., Cohen, P.A., Gendler, S.J., Boons, G.J., 2012. Immune recognition of tumor-associated mucin MUC1 is achieved by a fully synthetic aberrantly glycosylated MUC1 tripartite vaccine. Proc. Natl. Acad. Sci. U. S. A. 109, 261–266.
Langkilde, N.C., Wolf, H., Clausen, H., Kjeldsen, T., Orntoft, T.F., 1992. Nuclear volume and expression of T-antigen, sialosyl-Tn-antigen, and Tn-antigen in carcinoma of the human bladder. Relation to tumor recurrence and progression. Cancer 69, 219–227.
Leivonen, M., Nordling, S., Lundin, J., von, B.K., Haglund, C., 2001. STn and prognosis in breast cancer. Oncology 61, 299–305.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.
Marcos, N.T., Bennett, E.P., Gomes, J., Magalhaes, A., Gomes, C., David, L., Dar, I., Jeanneau, C., DeFrees, S., Krustrup, D., Vogel, L.K., Kure, E.H., Burchell, J., Taylor-Papadimitriou, J., Clausen, H., Mandel, U., Reis, C.A., 2011. ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues. Front. Biosci. (Elite. Ed.) 3, 1443–1455.
Marcos, N.T., Pinho, S., Grandela, C., Cruz, A., Samyn-Petit, B., Harduin-Lepers, A., Almeida, R., Silva, F., Morais, V., Costa, J., Kihlberg, J., Clausen, H., Reis, C.A., 2004. Role of the human ST6GalNAc-I and ST6GalNAc-II in the synthesis of the cancer-associated sialyl-Tn antigen. Cancer Res. 64, 7050–7057.
Margulis, V., Lotan, Y., Karakiewicz, P.I., Fradet, Y., Ashfaq, R., Capitanio, U., Montorsi, F., Bastian, P.J., Nielsen, M.E., Muller, S.C., Rigaud, J., Heukamp, L.C., Netto, G., Lerner, S.P., Sagalowsky, A.I., Shariat, S.F., 2009. Multi-institutional validation of the predictive value of Ki-67 labeling index in patients with urinary bladder cancer. J. Natl. Cancer Inst. 101, 114–119.
Miles, D., Roche, H., Martin, M., Perren, T.J., Cameron, D.A., Glaspy, J., Dodwell, D., Parker, J., Mayordomo, J., Tres, A., Murray, J.L., Ibrahim, N.K., 2011. Phase III multicenter clinical trial of the sialyl-TN (STn)-keyhole limpet hemocyanin (KLH) vaccine for metastatic breast cancer. Oncologist 16, 1092–1100.
Neutsch, L., Eggenreich, B., Herwig, E., Marchetti-Deschmann, M., Allmaier, G., Gabor, F., Wirth, M., 2012. Lectin bioconjugates trigger urothelial cytoinvasion – a glycotargeted approach for improved intravesical drug delivery. Eur. J. Pharm. Biopharm. 82, 367–375.
Numa, F., Tsunaga, N., Michioka, T., Nawata, S., Ogata, H., Kato, H., 1995. Tissue expression of Sialyl Tn antigen in gynecologic tumors. J. Obstet. Gynaecol. (Tokyo 1995) 21, 385–389.
Ogata, S., Koganty, R., Reddish, M., Longenecker, B.M., Chen, A., Perez, C., Itzkowitz, S.H., 1998. Different modes of sialyl-Tn expression during malignant transformation of human colonic mucosa. Glycoconj. J. 15, 29–35.
Ohno, S., Ohno, Y., Nakada, H., Suzuki, N., Soma, G., Inoue, M., 2006. Expression of Tn and sialyl-Tn antigens in endometrial cancer: its relationship with tumor-produced cyclooxygenase-2, tumor-infiltrated lymphocytes and patient prognosis. Anticancer Res. 26, 4047–4053.
Ozaki, H., Matsuzaki, H., Ando, H., Kaji, H., Nakanishi, H., Ikehara, Y., Narimatsu, H., 2012. Enhancement of metastatic ability by ectopic expression of ST6GalNAcI on a gastric cancer cell line in a mouse model. Clin. Exp. Metastasis 29, 229–238.
Palmeira, C., Lameiras, C., Amaro, T., Lima, L., Koch, A., Lopes, C., Oliveira, P.A., Santos, L., 2011. CIS is a surrogate marker of genetic instability and field carcinogenesis in the urothelial mucosa. Urol. Oncol. 29, 205–211.
Pinho, S., Marcos, N.T., Ferreira, B., Carvalho, A.S., Oliveira, M.J., Santos-Silva, F., Harduin-Lepers, A., Reis, C.A., 2007. Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett. 249, 157–170.
Pinto, R., Carvalho, A.S., Conze, T., Magalhaes, A., Picco, G., Burchell, J.M., Taylor-Papadimitriou, J., Reis, C.A., Almeida, R., Mandel, U., Clausen, H., Soderberg, O., David, L., 2012. Identification of new cancer biomarkers based on aberrant mucin glycoforms by in situ proximity ligation. J. Cell Mol. Med. 16, 1474–1484.
Ploeg, M., Aben, K.K., Kiemeney, L.A., 2009. The present and future burden of urinary bladder cancer in the world. World J. Urol. 27, 289–293.
Reis, C.A., Osorio, H., Silva, L., Gomes, C., David, L., 2010. Alterations in glycosylation as biomarkers for cancer detection. J. Clin. Pathol. 63, 322–329.
Ryan, S.O., Turner, M.S., Gariepy, J., Finn, O.J., 2010. Tumor antigen epitopes interpreted by the immune system as self or abnormal-self differentially affect cancer vaccine responses. Cancer Res. 70, 5788–5796.
Sandmaier, B.M., Oparin, D.V., Holmberg, L.A., Reddish, M.A., MacLean, G.D., Longenecker, B.M., 1999. Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cell rescue, and immunization with Theratope STn-KLH cancer vaccine. J. Immunother. 22, 54–66.
Santos, L., Amaro, T., Costa, C., Pereira, S., Bento, M.J., Lopes, P., Oliveira, J., Criado, B., Lopes, C., 2003. Ki-67 index enhances the prognostic accuracy of the urothelial superficial bladder carcinoma risk group classification. Int. J. Cancer 105, 267–272.
Schmitt, F.C., Figueiredo, P., Lacerda, M., 1995. Simple mucin-type carbohydrate antigens (T, sialosyl-T, Tn and sialosyl-Tn) in breast carcinogenesis. Virchows Arch. 427, 251–258.
Sewell, R., Backstrom, M., Dalziel, M., Gschmeissner, S., Karlsson, H., Noll, T., Gatgens, J., Clausen, H., Hansson, G.C., Burchell, J., Taylor-Papadimitriou, J., 2006. The ST6GalNAc-I sialyltransferase localizes throughout the Golgi and is responsible for the synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer. J. Biol. Chem. 281, 3586–3594.
Sievert, K.D., Amend, B., Nagele, U., Schilling, D., Bedke, J., Horstmann, M., Hennenlotter, J., Kruck, S., Stenzl, A., 2009. Economic aspects of bladder cancer: what are the benefits and costs? World J. Urol. 27, 295–300.
Sorensen, A.L., Reis, C.A., Tarp, M.A., Mandel, U., Ramachandran, K., Sankaranarayanan, V., Schwientek, T., Graham, R., Taylor-Papadimitriou, J., Hollingsworth, M.A., Burchell, J., Clausen, H., 2006. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. Glycobiology 16, 96–107.
Terashima, S., Takano, Y., Ohori, T., Kanno, T., Kimura, T., Motoki, R., Kawaguchi, T., 1998. Sialyl-Tn antigen as a useful predictor of poor prognosis in patients with advanced stomach cancer. Surg. Today 28, 682–686.
van der Velden, V.H., Hochhaus, A., Cazzaniga, G., Szczepanski, T., Gabert, J., van Dongen, J.J., 2003. Detection of

minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 17, 1013–1034.

van Rhijn, B.W., Burger, M., Lotan, Y., Solsona, E., Stief, C.G., Sylvester, R.J., Witjes, J.A., Zlotta, A.R., 2009. Recurrence and progression of disease in non-muscle-invasive bladder cancer: from epidemiology to treatment strategy. Eur. Urol. 56, 430–442.

Vazquez-Martin, C., Cuevas, E., Gil-Martin, E., Fernandez-Briera, A., 2004. Correlation analysis between tumor-associated antigen sialyl-Tn expression and ST6GalNAc I activity in human colon adenocarcinoma. Oncology 67, 159–165.

Videira, P.A., Calais, F.M., Correia, M., Ligeiro, D., Crespo, H.J., Calais, F., Trindade, H., 2009a. Efficacy of bacille Calmette-Guerin immunotherapy predicted by expression of antigen-presenting molecules and chemokines. Urology 74, 944–950.

Videira, P.A., Correia, M., Malagolini, N., Crespo, H.J., Ligeiro, D., Calais, F.M., Trindade, H., Dall'Olio, F., 2009b. ST3Gal.I sialyltransferase relevance in bladder cancer tissues and cell lines. BMC Cancer 9, 357.

Videira, P.A., Ligeiro, D., Correia, M., Trindade, H., 2007. Gene expression analysis in superficial bladder cancer: comparison of two suitable endogenous reference genes. Curr. Urol. 1, 145–150.

Yamamoto, M., Yamamoto, F., Luong, T.T., Williams, T., Kominato, Y., 2003. Expression profiling of 68 glycosyltransferase genes in 27 different human tissues by the systematic multiplex reverse transcription-polymerase chain reaction method revealed clustering of sexually related tissues in hierarchical clustering algorithm analysis. Electrophoresis 24, 2295–2307.

Yates, D.R., Roupret, M., 2011. Contemporary management of patients with high-risk non-muscle-invasive bladder cancer who fail intravesical BCG therapy. World J. Urol. 29, 415–422.

Zeichner, S.B., 2012. The failed Theratope vaccine: 10 years later. J. Am. Osteopath. Assoc. 112, 482–483.

3.2 Effect of mTor inhibitors on bladder cancer cells

Recently it has been reported that mTOR inhibitors could have an important role to play in bladder-cancer treatment and may restore chemosensitivity in resistant tumours

3.2.1 Paper IV

Everolimus combined with cisplatin has a potential role in treatment of urothelial bladder cancer.

Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, Colaço B, Lopes C, Colaço A, **Costa C**, da Silva VM, Oliveira P, Santos L.

Biomed Pharmacother. 2013. Mar; 67(2):116-21. doi: 10.1016/j.biopha.2012.11.007.

Biomedicine & Pharmacotherapy 67 (2013) 116–121

Available online at SciVerse ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com/en

Original article

Everolimus combined with cisplatin has a potential role in treatment of urothelial bladder cancer

Rosário Pinto-Leite [a,1], Regina Arantes-Rodrigues [b,2], Carlos Palmeira [c,d,3], Bruno Colaço [b,4], Carlos Lopes [e,5], Aura Colaço [b,6], Céu Costa [d,7], Vítor Moreira da Silva [f,3], Paula Oliveira [b,*], Lúcio Santos [c,3]

[a] Genetic Service, Cytogenetic Laboratory, Hospital Center of Trás-os-Montes and Alto Douro, 5000-508, Vila Real, Portugal
[b] Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, 5001-801, Vila Real, Portugal
[c] Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, 4200-072, Porto, Portugal
[d] Health Faculty, Fernando Pessoa University, 4200-1250, Porto, Portugal
[e] Department of Molecular Biology and Immunology, ICBAS, Porto University, 4099-003, Porto, Portugal
[f] Department of Urology, Portuguese Institute of Oncology, 4200-072, Porto, Portugal

ARTICLE INFO

Article history:
Received 8 November 2012
Accepted 23 November 2012

Keywords:
Bladder-cancer cell lines
Cisplatin
Everolimus

ABSTRACT

Cisplatin (CDDP)-based chemotherapy is a commonly treatment for advanced urothelial carcinoma. However, episodes of cisplatin resistance have been referenced. Recently it has been reported that everolimus (RAD001) could have an important role to play in bladder-cancer treatment and that mTOR inhibitors may restore chemosensitivity in resistant tumours. The aim of this study was to assess RAD001 in vitro ability to enhance CDDP cytotoxicity in three human bladder-cancer cell lines. Over the course of 72 h, the cells were exposed to different concentrations of CDDP and RAD001, isolated or combined. Treatment with CDDP statistically ($P < 0.05$) decreased cell proliferation in cell lines in a dose-dependent manner. The anti-proliferative activity of CDDP used in combination with RAD001 was statistically significant ($P < 0.05$) in the cell lines at all concentrations tested. RAD001 had a therapeutic effect when used in combination with CDDP and could therefore be a useful anti-cancer drug combination for patients with bladder cancer.

1. Introduction

The anti-tumour activity attributed to cis-diamminedichloroplatinium (II), best known as cisplatin (CDDP), is due to the fact it is an alkylating agent able to form a platinum complex, which results in DNA adducts. Consequently, intrastrand and interstrand DNA cross-links are induced. These result in DNA breaks and errors in DNA synthesis, with accumulation at the G_2 phase of the cell cycle leading to apoptosis [1–3]. Combined therapy of CDDP with other drugs such as methotrexate, vinblastine and doxorubicin or gemcitabine, is still one of the most-used therapeutic regimes for patients with metastatic bladder cancer [4,5]. Although there is a good rate of response to treatment (70%), a great majority of these cases (between 80% and 90%) develop recurrences and die [6,7]. CDDP was evaluated in several phase II and III studies associated with other drugs in the form of taxanes or triplets (such as paclitaxel conjugated with CDDP and gemcitabine), or in sequential protocols on patients with bladder cancer. These combined studies proved to be more effective, however they are also toxic [8] and bladder-cancer cisplatin resistance has been reported [9].

Everolimus (RAD001) is an immunosuppressive macrolide and an orally active rapamycin analogue that blocks the mammalian target of rapamycin (mTOR) signalling pathway, which is known to play an important role in protein synthesis, cellular metabolism and cell survival, thus inducing cell proliferation and angiogenesis [10–12]. RAD001's anti-cancer proprieties have been subject to evaluation using several tumour cell lines [13–15] and animal cancer models [13,16]. It has recently been reported that the PI3K/AKT pathway could be of importance in bladder cancer, and Chiong and collaborators verified that RAD001 inhibited the growth of bladder-cancer cells [13]. CDDP's efficacy when combined with everolimus has not yet been tested in bladder-cancer cell lines, and

* Corresponding author. Tel.: +35 19 66 47 30 62; fax: +35 12 59 35 04 80.
E-mail addresses: mlleite@chtmad.min-saude.pt (R. Pinto-Leite), arantesregina@hotmail.com (R. Arantes-Rodrigues), palmeira.ca@gmail.com (C. Palmeira), bcolaco@utad.pt (B. Colaço), calopes@icbas.up.pt (C. Lopes), acolaco@utad.pt (A. Colaço), ccosta@ufp.edu.pt (C. Costa), vitormoreira.silva@gmail.com (V.M. da Silva), pamo@utad.pt (P. Oliveira), llarasantos@gmail.com (L. Santos).
[1] Tel.: +35 12 59 30 05 00; fax: +35 12 59 30 03 47.
[2] Tel.: +35 19 35 60 77 83; fax: +35 12 59 35 04 80.
[3] Tel.: +35 12 25 08 40 00; fax: +35 12 25 08 40 01.
[4] Tel.: +35 19 39 45 37 04; fax: +35 12 59 35 04 80.
[5] Tel.: +35 12 25 51 11 94; fax: +35 12 25 51 11 94.
[6] Tel.: +35 19 34 28 70 04; fax: +35 12 59 35 04 80.
[7] Tel.: +35 12 25 07 46 30; fax: +35 12 25 07 46 37.

0753-3322/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biopha.2012.11.007

research into a possible role for mTOR inhibitors in restoring chemosensitivity in resistant tumours is in progress [17].

The aim of this study was to assess RAD001's in vitro ability to enhance CDDP cytotoxicity in three human bladder-cancer cell lines.

2. Materials and methods

2.1. Chemicals

RPMI 1640 culture medium and trypsin were purchased from PAA (Austria). Fetal calf serum, penicillin and streptomycin were obtained from Biological Industries (Israel). L-glutamine, dimethylsulfoxide and RAD001 were provided by Sigma Aldrich (EUA). CDDP was obtained by Teva Pharma (Portugal). The 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (USA). Propidium iodide was obtained from Cytognos (Spain). In situ cell-death detection kit fluorescein was acquired from Roche (USA). Antibody Ki-67 was purchased from DAKO (Portugal) and 3,3-diaminobenzidine chromogen (DAB) was obtained from Zytomed Systems (Portugal). The secondary antibody is an Ultravision detection System kit from Lab Vision Corporation (Portugal).

2.2. Cell culture

The study was performed on the 5637 non-muscle invasive urothelial bladder-cancer cell line and on two established human invasive bladder-cancer cell lines: T24 and HT1376 (T24 cell line was provided by DSMZ, Düsseldorf, Germany; 5637 and HT1376 cell lines were kindly provided by Dr. Paula Videira of the Universidade Nova de Lisboa, Lisboa, Portugal).

Cells were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. The cell lines were cultured as a monolayer at 37 °C in 5% CO_2 in a humidified atmosphere and the cells were routinely subcultured by trypsinization.

Bladder-cancer cell lines were treated with CDDP (0, 1, 2, 3, 4 and 5 μg/ml) and with graded doses of RAD001 in concentrations ranging from 0 to 2 μM (0, 0.05, 0.1, 1 and 2 μM) for 72 h to assess dose-response profiles. Both drugs were freshly prepared before each experiment.

For the combination therapy, the concentration of CDDP used was obtained by the average IC_{50} dose (2.5 μg/ml) in all of the three cell lines. In these experiments, cells were exposed to CDDP in combination with 0.05, 0.1, 1 and 2 μM of RAD001 over the course of 72 h. Control samples were treated similarly but the culture medium was used without the drug.

2.3. Cytotoxicity assay

T24, 5637 and HT1376 cells were seeded in 96-well flat-bottomed micro titer plates (Sarstedt, USA) at a density of 2–3 × 10^4 cells/ml, based on the growth characteristics of each cell line, and treated with CDDP (0, 1, 2, 3, 4 and 5 μg/ml) and RAD001 (0, 0.05, 0.1, 1 and 2 μM) for 72 h. 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye solution (5 mg/ml) was added to each well and 4 h later the formazan crystals generated were dissolved in 100 μl of dimethylsulfoxide. The absorbance value of each well was measured on an automatic ELISA plate reader (Multiskan EX, Labsystems, Portugal) at 492 nm. After blank correction (medium containing tetrazolium dye without cells) the percentage of cell proliferation was determined as follows: $(Absorbance_{exp\ group}/Absorbance_{control}) \times 100$ [18]. Absorbance obtained from untreated cells was taken as 100% cell proliferation. Each experiment was done in triplicate for each drug concentration and all assays were repeated in three independent experiments.

2.4. Cell-cycle analysis

Flow cytometric measurements were carried out on a Coulter flow cytometer (EPICS™ XL-MCL™, Brea, USA). Data, normally from 1 × 10^6 cells, was acquired and analyzed using Modfit LT 3.0 software (Verity™). Cells were fixed in ice-cold ethanol 70%, for a minimum period of 1 h. Propidium iodide was added in order to label total cellular DNA, and the DNA-content histograms obtained were analyzed in order to evaluate the percentage of cells in each of the cell-cycle phases: G_0/G_1, S and G_2/M. All experiments were performed in triplicate.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay (TUNEL)

Cells (2 × 10^4 cells/ml) were seeded and allowed to attach overnight. After treatment with CDDP (2.5 μg/ml) and RAD001 (2 μM) for 72 h, in isolation or combined, apoptosis was detected by means of the fluorescein in situ cell-death detection kit, in accordance with the manufacturer's instructions. In brief, nuclei were counterstained with 4′,6-diamidino-2-phenylindol and fragmentation was detected by it turning a green colour. Positive (cells incubated with DNase I recombinant) and negative (cells incubated with all reagents except TdT) controls were included in each experiment. Slides were observed using a fluorescence microscope (Nikon Eclipse E400, Japan). The number of cells undergoing apoptosis was observed in random fields in each slide and 200 cells were counted. Cells were defined as apoptotic if the nuclear area of cells was positively labelled (green colour). The apoptotic index was defined as (number of apoptotic cells/total number of cells) × 100 [19].

2.6. Immunocytochemistry

Immunocytochemistry assay was performed in order to determine the Ki-67 expression. Cells were plated in 24-well chamber slides (Sarstedt, USA) and incubated overnight. Drugs were applied for 72 h, in isolation or combined. After being washed in phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed three times in an isotonic PBS buffer. Permeabilization was carried out using 1% Triton X-100 in PBS for 20 min at room temperature and internal peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. The primary anti-Ki-67 antibody (MIB-1, 1:100) was incubated for 1 hour at room temperature. After incubation of the secondary antibody, positive immunoreactivity was visible by means of the reaction of 3,3-diaminobenzidine chromogen. The samples were washed with water and contrasted with hematoxylin. Negative controls were performed by replacing primary antibody with PBS. Samples were analyzed with a Leica microscope under 200× original magnification.

2.7. Statistical analysis

An equality of variances was tested by using the Levene F test and the statistical significance of differences between treatments and the control group was determined by Dunnett's multiple comparison post-hoc test for the MTT assay. The Pearson product-moment correlation coefficient was used to evaluate the correlation (linear dependence) of the cell cycle with CDDP and RAD001. Significant immunocytochemical differences were analyzed by Chi-square test. Data was analyzed using SPSS 17.0 statistical

software (SPSS Inc. USA) and values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Cytotoxic effects of CDDP and RAD001 against HT1376, T24 and 5637 cells

The cell proliferation of the three cell lines studied was evaluated by MTT assay. Treatment with CDDP for 72 h statistically decreased proliferation in all the three cell lines in a dose-dependent manner ($P < 0.05$). In the presence of increasing doses of CDDP (0, 1, 2, 3, 4 and 5 μg/ml), the cell-proliferation rates of HT1376 started at approximately 86% and with 5 μg/ml it was 20%; on T24 it ranged between 72% and 42%; and on 5637 the variation was between 56% and 12% (Fig. 1A). The average IC_{50} of this drug across the three cell lines was 2.5 μg/ml.

As far as RAD001 cytotoxicity was concerned, the highest dose (2 μM) did not induce significant cell death in T24 and HT1376 cell lines, with the 5637 cell line being the most sensitive, showing approximately 30% growth inhibition at 1 μM (IC_{30}) (statistically significant at all concentrations when compared with the control group, $P < 0.05$) (Fig. 1B), data published in previous reports [20,21].

CDDP and RAD001 were examined during a simultaneous time period (CDDP at 2.5 μg/ml and 0.05, 0.1, 1 and 2 μM RAD001 concentration). The anti-proliferative activity of the conjugated treatment was statistically significant in all three cell lines at all concentrations tested, when compared with the untreated cells ($P < 0.05$). The T24 cell line was more resistant to drugs used in association, as documented by survival rates between 32.15% to 34.9%, when compared with the HT1376 (27 to 4.1%) and 5637 (10.7 to 6.6%) cancer cell lines (Fig. 1C).

3.2. Cell-cycle analysis

The pattern of cell distribution through the various phases of the cell cycle was different in the three cell lines studied and depending on the drug treatment applied (Table 1). At the 2.5 μg/ml CDDP, most T24 and HT1376 cells stopped at G_2/M phase (54.1% and 62.2%, respectively), while T24 untreated cells were predominantly at G_0/G_1 phase (92.8%) and HT1376 untreated cells were more evenly distributed throughout the cell cycle (G_0/G_1: 34.8%, S: 31.4%, G_2/M: 34.4%). An S phase arrest was detected on the 5637 cell line (89%). However, cell-cycle analysis using the software's synchronization wizard application revealed that this accumulation occurred in the last compartment of the S phase, in late S/G_2 phases.

Comparative analysis of the percentage of cells in each cell-cycle phase, with the untreated cells on one side and the different RAD001 concentrations tested, on the other, is summarized in Table 1. Cell-cycle alterations were only observed in the 5637 cell line, namely a decrease in the percentage of cells in S phase fraction ($P = 0.029$). This effect on the proliferative rate was inversely correlated with drug concentration ($r = -0.978$; $P = 0.004$).

In the combined drug schedule, a low number of cycle cells (as observed in MTT assay) were distributed throughout the cell cycle, with no detection of a cell-cycle arrest in a specific phase (Table 1).

3.3. TUNEL assay

In untreated cell cultures, most of the nuclei were undamaged. After treatment with CDDP at 2.5 μg/ml, DNA fragmentation increased by an average of 16.5% in T24, 41.5% in 5637 and 37.9% in HT1376 cell lines. However, at the highest dose of RAD001, a minor increase in DNA damage was detected in 5637 (6.5%), compared to untreated cells (3%). Following 72 h of simultaneous treatment

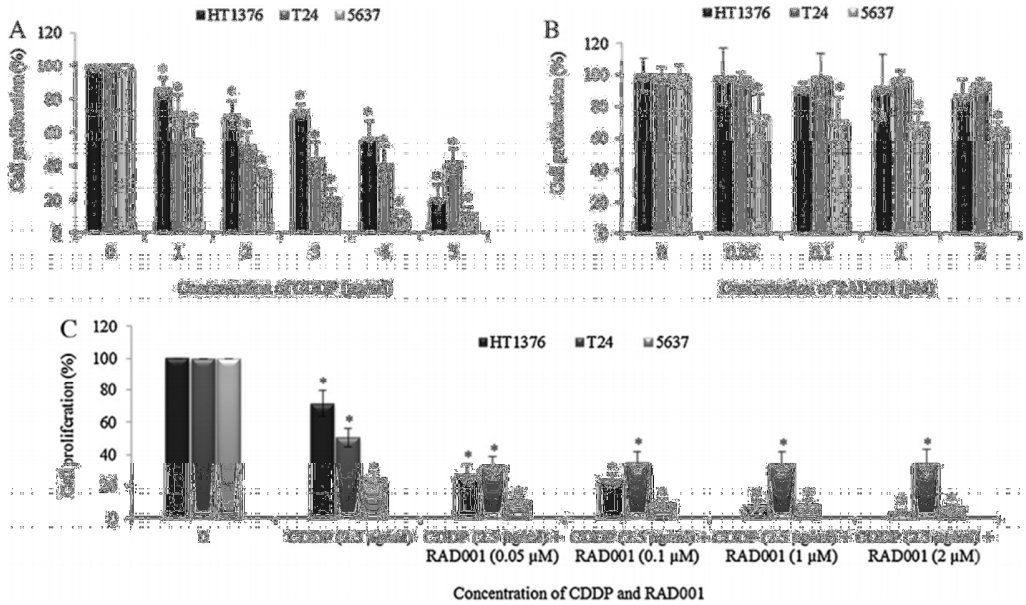

Fig. 1. Evaluation of cell proliferation on T24, 5637 and HT1376 human bladder cancer cell lines by MTT assay, after 72 h of drug exposure. A. With 0, 1, 2, 3, 4, and 5 μg/ml cisplatin (CDDP) exposure. B. With 0.05, 0.1, 1 and 2 μM everolimus (RAD001) exposure. C. Comparison of cell-poliferation percentages with 2.5 μg/ml CDDP in combination with 0.05, 0.1, 1 and 2 μM RAD001. The data shown and bars represent the mean value ± standard deviation. *$P < 0.05$ versus untreated cells.

Table 1
Cell cycle distribution on T24, 5637 and HT1376 cells after exposure to cisplatin (CDDP) and everolimus (RAD001), isolated or in combined schedule.

	T24			5637			HT1376		
	Cell cycle distribution (%)			Cell cycle distribution (%)			Cell cycle distribution (%)		
Treatment	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0	92.8	3.4	3.9	55.6	24.2	20.1	34.8	31.4	34.4
CDDP (2.5 μg/ml)	29	16.8	54.1	0.1	89	11	0.04	37.8	62.2
RAD001 (0.05 μM)	93.7	4.1	2.2	57.9	22.3	19.8	41.9	24.3	33.7
RAD001 (0.1 μM)	91.9	4.1	3.9	60.7	21.5	17.8	36.4	29.9	33.7
RAD001 (1 μM)	92.9	0.8	3.3	62	19.9	18	37.2	31	31.7
RAD001 (2 μM)	91.6	3.7	4.5	66.2	17	16.8	40.9	31.2	27.9
CDDP (2.5 μg/ml) + RAD001 (0.05 μM)	81	17	2	59.4	20.5	20.2	35.4	39.1	25.5
CDDP (2.5 μg/ml) + RAD001 (0.1 μM)	74.4	21.6	4	48.4	27.5	24.2	39.5	39.3	21.1
CDDP (2.5 μg/ml) + RAD001 (1 μM)	76.5	19.2	4.3	49	35.6	15.5	27	41.1	31.9
CDDP (2.5 μg/ml) + RAD001 (2 μM)	73.3	23	3.7	43.6	33.4	22.9	33.3	40.4	26.3

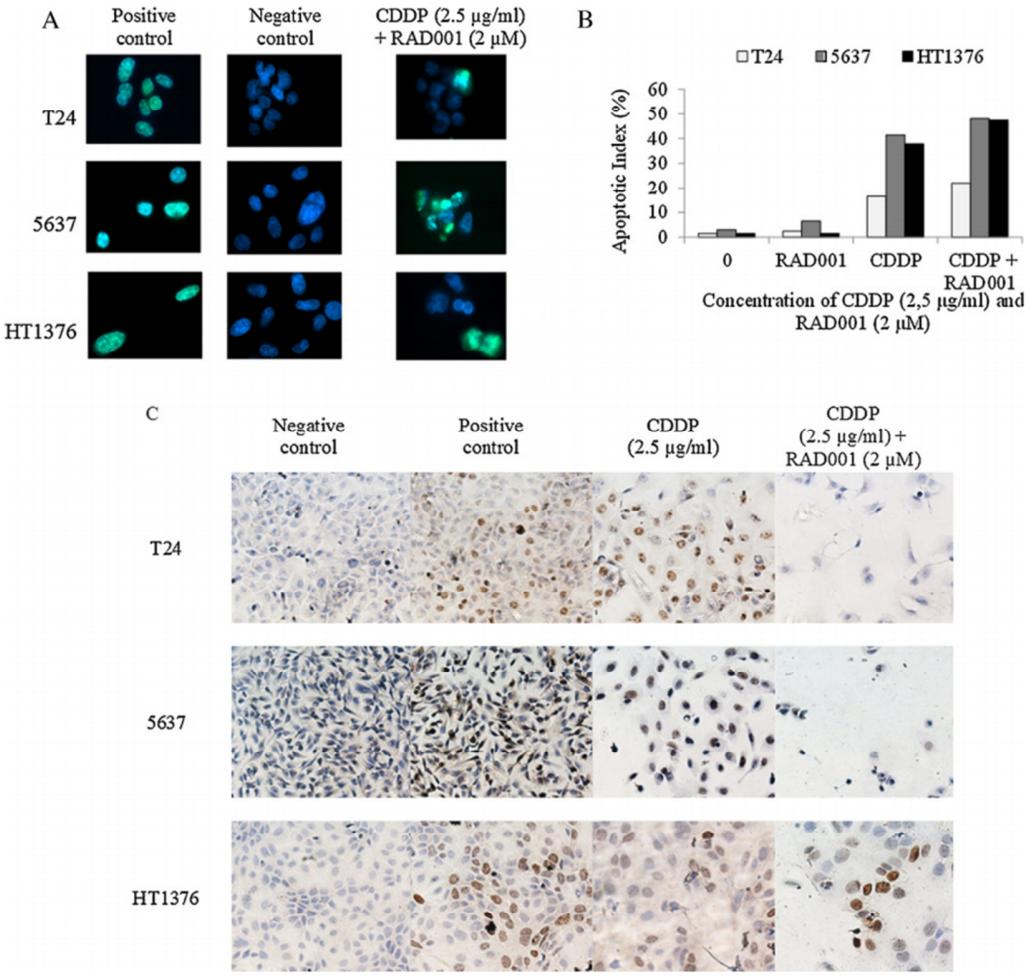

Fig. 2. A. Fluorescence images generated by TUNEL analysis with combined treatement on T24, 5637 and HT1376 bladder cancer cell lines, original magnification 1000×. B. Percentage of apoptotic cells after exposure to cisplatin (CDDP) and everolimus (RAD001), isolated or in simultaneous schedule. C. Ki-67 expression in bladder cancer cell lines treated with CDDP alone or in combination with RAD001 (original amplification 200×).

Table 2
Ki-67 immunocytochemical expression on T24, 5637 and HT1376 bladder cancer cell lines, treated with cisplatin (CDDP) and everolimus (RAD001), isolated or in simultaneous schedule.

Cell line	Control	CDDP	RAD001				CDDP + RAD001			
	0	2.5 μg/ml	0.05 μM	0.1 μM	1 μM	2 μM	CDDP (2.5 μg/ml) + RAD001 (0.05 μM)	CDDP (2.5 μg/ml) + RAD001 (0.1 μM)	CDDP (2.5 μg/ml) + RAD001 (1 μM)	CDDP (2.5 μg/ml) + RAD001 (2 μM)
T24	++++	++	+++	+++	+++	++	+	+	+	+
5637	++++	+++	+++	+++	+++	+++	–	–	–	–
HT1376	++++	+++	+++	+++	++	++	+	+	+	+

–: no staining; + very weak staining; ++: weak staining; +++: moderate to intense staining; ++++: highest intensity staining.

with CDDP (2.5 μg/ml) and RAD001 (2 μM), a considerable increase in apoptotic cells was observed, when compared to treatment with a single agent (at the same concentrations) or untreated cells. As shown in Fig. 2A and B, T24 tumour cell line showed less apoptotic cells (22%), in comparison with the HT1376 and 5637 cell lines in a conjugated experiment (47.5% and 48%, respectively).

3.4. Immunocytochemistry analysis

Immunocytochemistry was performed in order to evaluate Ki-67 expression in the three cell lines exposed to CDDP (2.5 μg/ml) and RAD001 (0.05, 0.1, 1 and 2 μM), in isolation or combined. All controls significantly expressed Ki-67 levels; while with CDDP and RAD001 there was also a moderate expression (Table 2). The combined schedule of both CDDP and RAD001 not only reduced the density of cells per microscope field, but it also reduced Ki-67 expression in those cell lines, although a slight Ki-67 expression in T24 and HT1376 cell lines was visible when compared with the 5637 cell line (Fig. 2C).

4. Discussion and conclusion

The purpose of our study was to evaluate if RAD001 could strengthen CDDP cytotoxicity using an in vitro model: a non-muscle invasive urothelial bladder-cancer cell line (5637) and two human invasive bladder-cancer cell lines (T24 and HT1376) that had not been evaluated previously. Combined therapy using CDDP and RAD001 has already being performed in pre-clinical studies, namely on advanced solid tumours [22], and it has also been studied in oesophageal [23], ovarian [24], nasopharyngeal [25] and non-small cell lung cancer [26] cell lines with encouraging effects and as a useful therapeutic strategy. Moreover, the benefits of RAD001 combined with other cytotoxic drugs were assessed as regards cisplatin resistance in patients with bladder cancer [27].

In our previous investigation, we found that isolated exposure of RAD001 on three human bladder-cancer cell lines showed heterogeneous results as far as cell proliferation and cell-cycle distribution were concerned, and we also evaluated RAD001's effect on the PI3K and MAPK pathway in these cell lines and its relationship with this relevant biological information [20,21]. CDDP exposition induced an effective decrease in cell proliferation in the three cell lines by MTT test and Ki-67 assay, cell-cycle arrest in the late S/G_2 phase, followed by a higher apoptotic rate detected by TUNEL assay.

Markedly increased cell cytotoxicity in the three cell lines was observed, when treated simultaneously with CDDP (at 2.5 μg/ml) and RAD001 in all the concentrations tested (0.05, 0.1, 1 and 2 μM). Also the Ki-67 assay analysis clearly demonstrated a reduction in the proliferation of the three cell lines. However, the T24 cell line showed a slightly different response pattern with a less intensive effect. The number of cells with DNA damage was higher when exposed to combined therapy than when single agents were used. Regarding cell-cycle analysis in the three bladder-cancer cell lines studied, cell-cycle arrest was not detected in any specific phase. Nevertheless, due to the effect of the combined drugs, the number of dividing cells found was much smaller and such cells suffered blocking at different stages – either in G_1/S or in G_2/M.

The distinct responses obtained in the three cell lines may reflect variability in bladder-cancer treatment that is eventually related to differences in chromosomal constitutions and correlated critical genes. As observed by Chiong and collaborators [13], despite the heterogeneity of responses seen in vitro, RAD001 is effective in vivo, because this drug affects tumour growth through different mechanisms, depending on the genotype of the bladder-cancer cells. The non-muscle invasive cancer cell line 5637 was the most sensitive to the action of the combined drug, thus opening a new line of research in this setting of bladder tumours. The anti-proliferative activity of CDDP in combination with RAD001 was statistically significant in the three cell lines, when compared with the cisplatin-treated cells. A similar trend was observed by Ma and collaborators (2010), which showed RAD001 exerted an additional synergistic effect on cisplatin-induced growth inhibition in CNE-1 and HONE-1 cells, and could inhibit the growth of cisplatin-resistant nasopharyngeal carcinoma cell lines [25]. It is necessary to evaluate this effect on bladder cancer, since the mTOR pathway was aberrantly activated in several bladder tumours [28]. Thus, it can be stated that RAD001 had a therapeutic effect when used in combination with cisplatin and this could be a useful anti-cancer drug combination for patients with bladder cancer.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

The authors thank the Portuguese Association of Urology and to FCT Pest-OE/AGR/UI0772/2011 unity.

References

[1] Bischin C, Lupan A, Taciuc V, Silaghi-Dumitrescu R. Interactions between proteins and platinum-containing anti-cancer drugs. Mini Rev Med Chem 2011;11:214–24.
[2] Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. Oncogene 2012;31(15):1869–83.
[3] Silva GN, Marcondes JPC, Camargo EA, Júnior GASP, Sakamoto-Hojo ET, Salvadori DMF. Cell cycle arrest and apoptosis in TP53 subtypes of bladder carcinoma cell lines treated with cisplatin and gemcitabine. Exp Biol Med 2010;235:814–24.
[4] Flaig TW, Su LJ, McCoach C, Li Y, Raben D, Varella-Garcia M, et al. Dual EGFR and VEGFR inhibition with vandetanib sensitizes bladder cancer cells to cisplatin in a dose and sequence dependent manner. BJU Int 2009;103(12):1729–37.
[5] Nordentoft I, Dyrskjøt L, Bødker JS, Wild PJ, Hartmann A, Bertz S, et al. Increased expression of transcription factor TFAP2a correlates with chemosensitivity in advanced bladder cancer. BMC Cancer 2011;11:135.

[6] Bellmunt J, Albiol S, Suárez C, Albanell J. Optimizing therapeutic strategies in advanced bladder cancer: update on chemotherapy and the role of targeted agents. Crit Rev Oncol Hematol 2009;69:211–22.
[7] Tatokoro M, Koga F, Yoshida S, Kawakami S, Fujii Y, Neckers L, et al. Potential role of Hsp90 inhibitors in overcoming cisplatin resistance of bladder cancer-initiating cells. Int J Cancer 2012;131(4):987–96.
[8] Ismaili N, Amzerin M, Flechon A. Chemotherapy in advanced bladder cancer: current status and future. J Hematol Oncol 2011;4:1–11.
[9] Drayton RM, Catto JW. Molecular mechanisms of cisplatin resistance in bladder cancer. Expert Rev Anticancer Ther 2012;12(2):271–81.
[10] Al-Batran SE, Ducreux M, Ohtsu A. mTOR as a therapeutic target in patients with gastric cancer. Int J Cancer 2012;130(3):491–6.
[11] Garcia JA, Danielpour D. mTOR inhibition as a therapeutic strategy in the management of urologic malignancies. Mol Cancer Ther 2008;7:1347–54.
[12] Rostaing L, Kamar N. mTOR inhibitor/proliferation signal inhibitors: entering or leaving the field? J Nephrol 2010;23:133–42.
[13] Chiong E, Lee IL, Dadbin A, Sabichi AL, Harris L, Urbauer D, et al. Effects of mTOR inhibitor everolimus (RAD001) on bladder cancer cells. Clin Cancer Res 2011;17:2863–73.
[14] Mansure JJ, Nassim R, Chevalier S, Rocha J, Scarlata E, Kassouf W. Inhibition of mammalian target of rapamycin as a therapeutic strategy in the management of bladder cancer. Cancer Biol Ther 2009;8:2339–47.
[15] Marinov M, Ziogas A, Pardo OE, Tan LT, Dhillon T, Mauri FA, et al. AKT/mTOR pathway activation and BCL-2 family proteins modulate the sensitivity of human small cell lung cancer cells to RAD001. Clin Cancer Res 2009;15:1277–87.
[16] Morgan TM, Pitts TE, Gross TS, Poliachik SL, Vessella RL, Corey E. RAD001 (Everolimus) inhibits growth of prostate cancer in the bone and the inhibitory effects are increased by combination with docetaxel and zoledronic acid. Prostate 2008;68(8):861–71.
[17] Gaur S, Chen L, Yang L, Wu X, Un F, Yen Y. Inhibitors of mTOR overcome drug resistance from topoisomerase II inhibitors in solid tumors. Cancer Lett 2011;311(1):20–8.
[18] Mabuchi S, Altomare A, Cheung M, et al. RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. Clin Cancer Res 2007;13:4261–70.
[19] Tang ZY, Wu YL, Gao SL, Shen HW. Effects of the proteasome inhibitor bortezomib on gene expression profiles of pancreatic cancer cells. J Surg Res 2000;145:111–23.
[20] Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, Gaivão I, Cardoso ML, Colaço A, et al. Everolimus enhances gemcitabine-induced cytotoxicity in bladder-cancer cell lines. J Toxicol Environ Health A 2012;75(13–15):788–99.
[21] Vasconcelos-Nóbrega C, Pinto-Leite R, Arantes-Rodrigues R, Ferreira R, Brochado P, Cardoso ML, et al. In vivo and in vitro effects of RAD001 on bladder cancer. Urol Oncol 2011. http://dx.doi.org/10.1016/j.urolonc.2011.11.002.
[22] Fury MG, Sherman E, Haque S, Korte S, Lisa D, Shen R, et al. A phase I study of daily everolimus plus low-dose weekly cisplatin for patients with advanced solid tumors. Cancer Chemother Pharmacol 2012;69(3):591–8.
[23] Hirashima K, Baba Y, Watanabe M, Karashima RI, Sato N, Imamura Y, et al. Aberrant activation of the mTOR pathway and anti-tumour effect of everolimus on oesophageal squamous cell carcinoma. Br J Cancer 2012;106(5):876–82.
[24] Mabuchi S, Kawase C, Altomare DA, Morishige K, Sawada K, Hayashi M, et al. mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. Clin Cancer Res 2009;15:5404–13.
[25] Ma BB, Lui VW, Hui EP, Lau CP, Ho K, Ng MH, et al. The activity of mTOR inhibitor RAD001 (everolimus) in nasopharyngeal carcinoma and cisplatin-resistant cell lines. Invest New Drugs 2010;28:413–20.
[26] Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, O'Reilly T, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell 2005;120:747–59.
[27] Rexer H. Second line AUO (Working Group Urological Oncology) study AB 35/09 on metastasized urothelial carcinoma. Phase II study with RAD001 in combination with paclitaxel for patients with metastasized urothelial carcinoma of the urinary bladder after failure of platinum-based chemotherapy (CRAD001). Urologe A 2011;50(11):1464–588.
[28] Nawroth R, Stellwagen F, Schulz WA, et al. S6K1 and 4E-BP1 are independent regulated and control cellular growth in bladder cancer. PLoS One 2011;6(11):e27509.

3.3 Patient-derived bladder cancer xenografts

Patient-derived tumour xenografts (PDTXs) have been shown to be a highly predictive model to test standard chemotherapy and for identification of tumour types that might benefit from new treatments in clinical trials.

3.3.1 Paper V

Patient-derived bladder cancer xenografts: a systematic review.

Bernardo C, **Costa C**, Sousa N, Amado F, Santos L

Transl Res. 2015. Feb 12. pii: S1931-5244(15)00040-7. doi: 0.1016/j.trsl.2015.02.001.

REVIEW ARTICLE

Patient-derived bladder cancer xenografts: a systematic review

CARINA BERNARDO, CÉU COSTA, NUNO SOUSA, FRANCISCO AMADO, and LÚCIO SANTOS

PORTO AND AVEIRO, PORTUGAL

Patient-derived tumor xenografts (PDTXs) are said to accurately reflect the heterogeneity of human tumors. In the case of human bladder cancer, few studies are available featuring these models. The best methodology to develop and the real value of the model remain unclear. This systematic review aims to elucidate the best methodology to establish and use PDTXs to study the characteristics and behavior of human bladder tumors. The value and potential application of these models are also addressed. A comprehensive literature search was performed to identify published studies using xenograft models directly established from human bladder cancer samples into mice. A total of 12 studies were included in the final analysis. All studies differed in design; the reported take rate varied between 11% and 80%, with the implantation via dorsal incision and with matrigel obtaining the higher take rate. Advanced stage and high-grade tumors were associated with increased take rate. Xenografts preserved the original tumor identity in the establishment phase and after serial passages. Although some studies suggest a correlation between engraftment success and clinical prognosis, evidence about the association between the response of xenografts to treatment and the clinical response of the tumor of origin is still missing. All methodological approaches resulted in the establishment of tumor xenografts with preservation of the original tumor identity but variable take rate. The time needed to establish the model and propagate xenografts to a number suitable for drug testing is the main limitation of the model, along with the success rate and lack of consistency in the early passages. Comparison between tumor response in mice and clinical outcome remains to be assessed. (Translational Research 2015; ■:1–8)

Abbreviations: IP – inverted papilloma; NEBC – neuroendocrine bladder cancer; PDTX – patient-derived tumor xenografts; SCC – squamous cell carcinoma; SCID – severe combined immunodeficient; Small CC – small cell carcinoma; TCC – transitional cell carcinoma

From the Experimental Pathology and Therapeutics Group, Research Center, Portuguese Institute of Oncology Francisco Gentil, Porto, Portugal; Health Science Department (SACS), University of Aveiro, Aveiro, Portugal; Biomedical Research Centre (CEBIMED), Health Sciences Faculty, Fernando Pessoa University, Porto, Portugal; Department of Medical Oncology, Portuguese Institute of Oncology Francisco Gentil, Porto, Portugal; QOPNA, School of Health Sciences, University of Aveiro, Aveiro, Portugal; Department of Surgical Oncology, Portuguese Institute of Oncology Francisco Gentil, Porto, Portugal.

Submitted for publication October 13, 2014; revision submitted February 4, 2015; accepted for publication February 6, 2015.

Reprint requests: Carina Bernardo, Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology Francisco Gentil, Rua Dr António Bernardino de Almeida, 4200-072 Porto, Portugal; e-mail: carinadbernardo@gmail.com.

1931-5244/$ - see front matter

http://dx.doi.org/10.1016/j.trsl.2015.02.001

INTRODUCTION

Bladder cancer is the second most common genitourinary tumor, with about 386,300 new cases and 150,200 deaths estimated to have occurred in 2008 worldwide.[1] Most of these cases, approximately 75%, are nonmuscle invasive tumors for which local treatment with excision and adjuvant intravesical immunotherapy or chemotherapy are associated with high cure rates. Nevertheless, 10%–20% of these superficial tumors will recur and progress to invasive disease. Patients with muscle invasive bladder cancer have a poor prognosis with low 5-year survival rate.[2] In these cases, treatment may include cystectomy and chemotherapeutic regimens containing cisplatin.[3] The low overall response rate and unpredictable outcome for these patients have led to the search for reliable tools for assessment of prognosis and indications for therapy. The development of predictive tumor models may be a valuable aid to this purpose.

Patient-derived tumor xenografts (PDTXs) have been shown to be a highly predictive model to test standard chemotherapy and for identification of tumor types that might benefit from new treatments in clinical trials.[4] This model is established by transferring fragments of tumor derived from an individual patient into immunocompromised animals such as severe combined immunodeficient (SCID) or nude mice. Because they derive directly from patient tumor samples with minimal manipulation, the xenografts retain the cellular structure and molecular markers of the original tumors.[5] Therefore, tumor xenografts recapitulate the biological characteristics of the disease of origin and are suitable for evaluation of an individual patient's cancer chemosensitivity, providing not only an investigational platform but a potential therapeutic decision-making tool.[6-8]

Human tumor xenograft models have been developed for several types of cancers, such as lung, prostate, breast, liver, and colon carcinomas as tools for evaluation of new therapeutic strategies or individualization of cancer treatment.[7-11] However, in the case of human bladder cancer, few studies of PDTXs are available. Furthermore, to optimize and standardize the use of these models, it is important to assess if such studies clearly report the methodology and results in a way that internal reproducibility can be assessed and if the results can be systematically compared with other studies.

In this context, we conducted this systematic review on PDTX of bladder cancer to elucidate the best methodology to establish and use such models to study the characteristics and behavior of human bladder tumors.

METHODS

Search strategy. All potentially relevant articles were identified by searches done via PubMed on the Medline database using the following search terms in title or abstract: transitional cell carcinoma OR transitional cell carcinomas OR urothelial tumor OR urothelial cancer OR urothelial carcinoma OR urothelial carcinomas OR bladder tumor OR bladder cancer OR bladder cancers OR bladder carcinoma OR bladder carcinomas AND xenograft OR xenografts OR xenotransplant and the following MeSH term: animal experimentation. All relevant articles published up to February 2014 were selected and no limits were introduced for the search strategy. Furthermore, this electronic search was complemented by hand searching the references listed in any included study.

Eligibility criteria. The following criteria had to be met for a study to be included in this review: xenograft model directly established from human bladder cancer samples into mice, regardless of bladder cancer grade or stage. References not written in English or those that were reviews, editorials, or letters were excluded. Any selected reference for which a full text report was not available after contact with dedicated libraries or with the corresponding author was also excluded.

All retrieved references were screened for eligibility based on title and abstract review by 2 of the authors. All references deemed potentially eligible were retrieved for full text assessment of eligibility by 2 of the authors.

Data extraction. Data extraction was performed by 2 authors with the use of a predefined data collection form. The following data were collected from each reference: article identification details (title, authors, and publication date); methodological details (study design, number and characteristics of mice used, and number of patients and primary tumor sample characteristics); tumor take rate (defined as the percentage of mice with xenograft growth divided by the total number of mice implanted, should more than 1 fragment be implanted per mouse into distinct locations, each was considered as independent study units for this end point); success rate (defined as the number of xenografts obtained per human tumor sample used); lag period; number of passages; xenograft histologic and molecular characteristics; and comparison of xenograft characteristics with the original human tumor. When analyzing tumor take rate and success rate, engraftment was considered successful when a progressive tumor growth in man-to-mouse generation passage reached a tumor volume of at least 100 mm^3. At this point, the growing tumor can be clearly identified and measured and the risk of spontaneous regression is low.

Data analysis. The primary end point chosen for this review was tumor take rate as it reflects the efficacy of the method used to establish the xenograft. In this review, only articles with complete information about the number of mice implanted per tumor sample and number of fragments implanted per mice in independent sites were considered to calculate the take rate. An average tumor take rate was planned to be calculated from the retrieved data, if methodological homogeneity in the development of PDTXs was identified.

Secondary end points such as histologic, immunohistochemical, and genetic assessment were considered as a measurement of tumor identity preservation. Histologic evaluation and comparison of tissue architecture and cellular appearance between the xenografts and the corresponding original tumor were considered, as well as tumor marker expression, chromosome analysis, and genetic mutation analysis.

The focus of this review was on methodological variations that could influence the success rate and the preservation of tumor identity. Therefore, we planned to implement a qualitative systematic review of the data. However, additional data such as xenograft response to treatments were also inspected and if reported were to be collected.

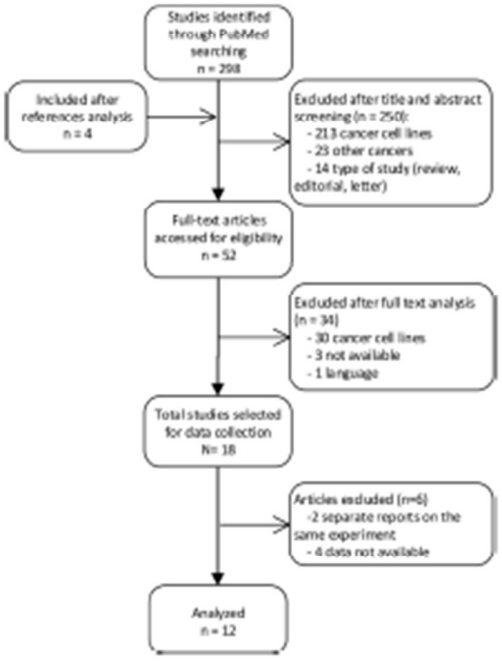

Fig 1. Flow diagram of the systematic review process.

RESULTS

The systematic review process is represented in Fig 1. A total of 298 articles were identified through PubMed search, and hand searching the reference lists of included studies has increased the sampling basis by 4 references. Of those 302 total references, 251 articles were excluded after title and abstract screening and 32 excluded after full text analysis. The resulting 18 articles were analyzed and information extracted with a predefined data collection form by 2 authors. Of these, 2 articles were excluded for being separate reports on the same experiment at different time points[12-15] and 4 were excluded for not providing data about the methodology used or results.[16-19] A total of 12 studies were included in the final analysis.

All selected studies differed in design and many did not report details, thus making their systematic interpretation and evaluation a challenge. Early studies used immunocompetent mice subjected to immunosuppressive treatment before tumor xenotransplantation or T-cells deficient mice. Later on, highly immunodeficient mice such as SCID, nonobese diabetic SCID, and Rag strains became the most commonly used mice for xenograft development. Human tumors were implanted as fragments or after cells dissociation and xenograft growth evaluated with caliper measurements. The first studies on bladder cancer xenografts were mainly focused on the evaluation of xenograft phenotype after growing in mice but later, molecular and genetic analysis were also used to access xenograft identity.

Engraftment success. The preparation of tumor samples for implantation was quite similar among all studies. Immediately after specimen collection and before transplantation, the tumors were maintained in media alone, medium containing penicillin and streptomycin or sterile salt solution. The samples were then cut into 1–3 mm cubes for subcutaneous implantation through small dorsal incisions or using trocar needle. In one study, the tumor samples were submitted to mechanical and enzymatic dissociation before being subcutaneously injected as cellular suspension. The implantation of tumor fragments and inoculation of tumor cells was performed alone or with matrigel.

The tumor take rate was reported in 6 of 12 studies. It varied between 11% and 62% after tumor fragment implantation through small dorsal incision without matrigel and 80% with matrigel. In case of tumor implantation via needle injection, without matrigel, one study reported take rate of 19%.[20] One study reported a take rate of 36% after suspended and sorted tumor cells' implantation with matrigel.[21] Table I presents the tumor take rate obtained in the included studies considering tumor sample processing and method of implantation.

Table I. Tumor take rate after the first implantation in mice

Tumor sample	Matrigel	Implantation	Take rate	Study
Fragment				
	No	Injection	19% (10/52)	Matthews et al (1982)[20]
	No	Incision	48% (28/62)	Hay et al (1986)[22]
	No	Incision	62%	Abe et al (2006)[23]
	Yes	NA	80% (12/15)	Hofner et al (2013)[24]
	No	Incision	11% (1/9)	Bernardo et al (2014)[25]
Cell suspension				
	Yes	Injection	36% (5/14)	Chan et al (2009)[21]

Considering the reported take rate, tumor implantation through incision or by injection seems to be equally effective to obtain tumor growth; however, the association with matrigel seems to improve the take rate. On the other hand, enzymatic tumor dissociation and implantation of cellular suspension resulted in a take rate equivalent to that obtained with tumor fragment implantation.

Considering the success rate and the characteristics of the tumors implanted, higher stage and grade is associated with higher probability of successful xenograft establishment. The success rate and take rate of each study is presented in Table II, along with the classification of the primary tumors.

Preservation of tumor identity. Tumor identity preservation is reported in all studies with, at least, histologic examination after hematoxylin and eosin staining and comparison with the original tumor. Nine studies complement basic histologic examination through the use of immunohistochemistry assessment of specific tumor markers (5 studies) or genetic analysis (7 studies).

Preservation of tissue architecture and cellular appearance were reported by all studies after histologic analysis of the xenografts and comparison with the corresponding primary tumors. Exceptions were reported by Abe et al[23] in which 3 of the 15 xenografts established were different from the original tumor. The development of squamous features and some change toward a greater or lesser degree of differentiation were also reported as minor changes during serial passage.[14,15,22] The studies that evaluated the xenografts after several passages confirmed the histologic stability of the xenografts in later passages.[12,22,23] The xenograft molecular characterization is summarized in Table III.

Immunohistochemical assessment of xenografts was reported in 5 studies. The most frequently analyzed markers were P53 and Ki-67. When analyzed, P53 expression and mutational status were found concordant between the original tumor and the xenografts.[23,28] On the other hand, Ki-67 index was increased in xenografts comparing with the original tumor, particularly after serial passages in mice.[23,25] These studies found no statistically significant relationship between success take rate and malignant characteristics of the original tumor (P53, Ki-67).

Chromosome analysis was performed in 4 studies along different passages and showed human karyotype in all xenografts analyzed[12,14,22,26] Xenograft characterization through mutational analysis, short tandem repeats (STRs) genotyping, and array-comparative genomic hybridization were performed in one study.[29] In the 6 xenografts with 2 or more passages they found 2 completely identical STR profiles and 4 nearly identical profiles for all STR loci; 4 of 6 had identical mutations in the TP53, HRAS, BRAF, and CTNNB1 (catenin [cadherin-associated protein], beta 1) genes; and all 6 xenografts had genomic alterations similar to the original tumor samples.

Metastasis was not found in any of the studies; however, tumor invasion of the abdominal wall, nerves, and veins of the mouse was reported by Hay et al.[22]

DISCUSSION

Xenografts of human tumors have been considered a valuable and essential tool for the study of tumor biology and behavior and to evaluate new anticancer drugs before the initiation of human clinical trials.[4] Patient-derived xenografts (PDX) started to be used as a complement to the traditional tumor model system in the preclinical evaluation of new drugs, which were mainly based on established human cancer cell lines. The selective pressure undergone during the establishment and propagation of tumor cell lines lead to the acquisition of new characteristics and to the selection of subpopulations. Such events contribute to the modest predictive power of in vitro and in vivo models based on cell lines.[30] In the case of PDX, fragments of patient tumor samples, with minimal manipulation, are directly implanted in mice; these xenografts had shown to better retain the cellular structure and molecular markers of the original tumors.

PDTX models have been established for decades but only recently began to be consistently characterized and used in the evaluation and development of cancer therapies as reviewed elsewhere.[31] PDX models of human

Table II. Bladder cancer xenograft establishment efficiency and sample characteristics

Study features	Sutfin et al (1979)[26]	Nalto et al (1981)[27]	Matthews et al (1982)[20]	Kovnat et al (1982)[14]	Russell et al (1986)[12]	Hoy et al (1986)[22]	McCue et al (1996)[28]	Abe et al (2006)[23]	Chan et al (2009)[21]	Hofner et al (2013)[24]	Park et al (2013)[29]	Bernardo et al (2014)[25]	Average
N samples	20	31	18	33	20	48	17	24	14	2	65	1	**24**
Success rate	40%	8/31 (26%)	6/18 (33%)	8/33 (24%)	11/20 (55%)	7/48 (15%)	10/17 (59%)	15/24 (62.5%)	5/14 (35.7%)	2/2 (100%)	10/65 (15.4%)	1/1 (100%)	**47%**
Take rate	41/104* (39%)	NA†	10/52 (19.23%)	34.14%‡	NA	47.8%	NA	62.5%	35.7%	80%	NA	1.1%	**41%**
Histology	TCC	7/30 TCC, 1/1 SCC	TCC	TCC	TCC	TCC/mixed	TCC	12/20 TCC, 2/2 small CC, 1/1 SCC 0/1 IP	NA	NEBC	NA	TCC	—
Stage													
Ta		**4/19**	**2/6**	3	1	0	NA	**2/5**	**0/1**	**0**	**1/22**	**0**	**20%**
T1	(I–II)	**3/6**	**1/8**	2	3	0	NA	**4/8**	**0/0**	**0**	**5/22**	**0**	**34%**
T2	3/9	**0/3**	**3/4**	0	2	1	NA	**5/5**	**1/5**	**0**	**2/12**	**0**	**42%**
T3	(II–V)	**0/2**	**0**	2	1	5	NA	**3/3**	**4/6**	**1/1**	**1/4**	**1/1**	**65%**
T4	5/11	**1/1**	**0**	9	1	1	NA	**1/2**	**0/0**	**1/1**	**1/4**	**0**	**69%**
Tis			**0**	1	0	0	NA	**0**	**0/1**	**0**	**0/1**	**0**	**0%**
N/A									**0/1**				**0%**
Grade													
I	(I–II)	**0/5**	**3/9**	4	0	**0/8**	0/3§	**0/1**	**NA**	**0**	**0/2**	**0**	**7%**
II	6/9	**7/20**	**2/8**	2	4	**3/17**	5/8§	**4/7**	**NA**	**0**	**4/39**	**0**	**29%**
III	2/11	**1/6**	**1/1**	2	6	**4/21**	3/4§, 2/2§	**11/15**	**NA**	**2/2**	**6/24**	**1/1**	**60%**
Other	0	0	0	0	0	0/2	0	0/1	0	0	0	0	—
≥2 Passages	—	8	4	3	3	7	3	11	NA	2	7	1	—
Mouse strain	Athymic nude	BALB c nu/nu	Athymic nude	CBA/CBJ‖	BALB c nu/nu	CBA¶	CB17-SCID	CB17-SCID	Rag2-γc-	NOD SCID γ	Athymic nude	SCID, NINH (II)	—
Previous CTx or RTx	NA	NA	NA	NA	8 No, 3 yes	No	NA	NA	NA	No	2 No, 3 yes	No	—

Abbreviations: CTx, chemotherapy; P, Inverted papilloma; NEBC, neuroendocrine bladder cancer; NIH, National Institutes of Health; NOD, nonobese diabetic; RTx, radiotherapy; SCC, squamous cell carcinoma; SCID, severe combined immunodeficient; Small CC, small cell carcinoma; TCC, transitional cell carcinoma.

Tumor stage and grade: number of successful xenografts/total number of tumors implanted. Average was calculated based on the results in bold.

*Sutfin et al reported the take rate per animal implanted; however, mice were implanted in 3–4 different sites and were considered as 1 single unit.

†Nalto et al do not present the take rate of the first passage, only the take rate of all passages altogether.

‡Kovnat et al implanted 1 or 2 samples per mice but only reported the take rate per mice implanted, not per site.

§Not included in the average calculations. Different classification system.

‖Mice subjected to thymectomy + ara C treatment + irradiation prior tumor implantation.

Table III. Xenograft molecular characterization

Study	Immunohistochemistry	Gene analysis	Other
Sufrin et al (1979)[26]	—	—	Karyotype
Kovnat et al (1982)[14]	—	—	Karyotype
Hay et al (1986)[22]	—	—	Karyotype
Russell et al (1986)[12]	CEA, β-HCG, PNA	—	DNA flow cytometry, karyotype
McCue et al (1996)[28]	ckAE1/AE3, PCNA, HLA-A, H2, P53	—	DNA content
Abe et al (2006)[23]	CD20, Ki-67, LMP-1, HLA-ABC	P53	EBV, p53 functional assay
Hofner et al (2013)[24]	Ki-67, CD56, synaptophysin		
Park et al (2013)[29]		Mutations	STR genotyping, array-comparative genomic hybridization
Bernardo et al (2014)[25]	P53, Ki-67, p63, ck20, sTn	—	sTn WB

Abbreviations: CEA, carcinoembryonic antigen; EBV, Epstein-Barr virus; HCG, human chorionic gonadotropin; HLA-A, major histocompatibility complex, class I, A; HLA-ABC, human major histocompatibility complex, class I, HLA-A, B and C; LMP, latent membrane protein; PCNA, proliferating cell nuclear antigen; PNA, Peanut agglutinin.

cancers, such as prostate, non-small cells lung cancer, breast cancer, melanoma, and head and neck cancer have been developed and extensively characterized in terms of histologic, immunophenotypical, and genetic concordance with the original tumor and responsiveness to commonly used treatment agents in the clinic.[4,31] These models value are now widely recognized and their applications expanded.

The heterogeneity of the tumor samples implanted is regarded as an advantage over the xenograft models developed with established cancer cell lines as they better reflect the complexity of human tumor composition and development. However, the heterogeneous tumor samples include varying tumor cell contents, extracellular matrix, immune cells, and necrosis, which can also contribute to distinct tumor success rates and low reliability. The use of sorted cell populations or primary cell cultures can provide a more reproducible source of tumor material for preclinical model development as they reduce the heterogeneity of tumor cell population, but these artificial tumor simplifications can also have implications on the predictive value of the model. This approach has been used in recent studies in which models were used for very distinct purposes.[16-18,21]

Considering the studies on PDTX published till date, all methodological approaches resulted in the establishment of tumor xenografts with preservation of the original tumor identity but variable take rate. The time needed to establish the model and propagate xenografts to a number suitable for drug testing is the main limitation of the model, along with the success rate and lack of consistency. The success of the first implantation in mice is a critical step in the process. To increase the chances of success when optimizing the number of mice needed, multiple tumor fragments of 2–5 mm can be implanted per mice, through small incisions, in different sites (2–4). The incubation of the tumor samples with matrigel also seems to increase the take rate. On the other hand, additional tumor fragmentation needed for tumor injection using needle should be avoided, as it can cause more damage to the tumor tissue architecture with no apparent benefit on the success rate. The number of mice first implanted with the patient tumor varied depending on the amount of tumor sample available. Some studies started with one mouse per sample, other with 5–6 or even 9. However, with viable tumor sample and 2 implantation sites, 4 mice should be enough to obtain successful xenograft growth.

Serial xenograft passage is another essential aspect in the model development, as it is needed to achieve a sufficient number of grown tumors suitable for drug testing. All studies under review were able to successfully pass the xenografts to other mice, with the exception of Sufrin et al,[26] who stopped the study in the first passage. To evaluate tumor response to chemotherapy, 2 fragments of 2 mm can be implanted per mice in different sites and evaluated as 2 independent units, with 8–10 tumors per treatment condition. This approach was used to evaluate chemotherapy response of xenografts in pilot clinical study involving patients with advanced cancer.[7]

Large-scale human tumor xenograft programs have been reviewed and the activity of new agents in these models evaluated and compared with the activity of the drug in clinical trials.[32,33] The predictive power of xenografts varied according to the type of tumor, for example, the model showed good predictive value for lung and ovarian cancer, but failed to adequately predict in the case of breast and colon tumors.[33]

Although direct bladder cancer xenografts have shown to preserve tumor histology and molecular signature of the original tumor, the value of this model in predicting clinical results remains poorly investigated. No study assessed the clinical predictive power of its model. However, some studies report the correlation between engraftment success and clinical prognosis or pathologic stage and grade of the original tumor.

Comparison between tumor response in mice and clinical outcome remains to be assessed. Studies involving a larger number of patients, tumor sensitivity analysis, and longer patient follow-up may be useful to fully explore the potential and predictive value of this model. Another potential method to explore the predictive power of bladder cancer PDX is the use of genomic profiling as in the co-expression extrapolation (COXEN) model. The COXEN algorithm predicts the effectiveness of chemotherapeutic agents for cancer cell lines or tumor samples by relating their expression signatures to signatures of cell lines with known drug sensitivity.[34] Thus, the application of the COXEN model in bladder cancer xenografts would be of interest to evaluate the response of these tumors to chemotherapy.

Finally, some aspects need to be taken into consideration when using xenografts, the stroma and blood supply is provided by the host and the tumor is not growing in the organ of origin. This later aspect may be associated with the infrequent occurrence of metastatic disease in mice. In the case of bladder cancer, none of the studies analyzed in this review observed metastasis. On the other hand, it is also important to evaluate if the tumor clones selected by the process of xenograft establishment and propagation are somehow similar to the tumor cells responsible for tumor recurrence and metastasis in patients with bladder cancer.

ACKNOWLEDGMENTS

Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

C.B. thanks the FCT (Fundação para a Ciência e a Tecnologia) for the PhD scholarship SFRH/BD/80855/2011.

All authors contributed to the ideas and writing associated with the manuscript. It has been reviewed and approved by all named authors.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
2. Knowles M. What we could do now: molecular pathology of bladder cancer. Mol Pathol 2001;54:215–21.
3. Bellmunt J, Albiol S, Kataja V. Invasive bladder cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. Ann Oncol 2009;20(suppl 4):79–80.
4. Fiebig HH, Dengler AW, Roth T. Human tumor xenografts: predictivity, characterization and discovery of new anticancer agents. In: Fiebig HH, Burg AM, eds. Relevance of tumor models for anticancer drug development Contr Oncol Basel. Karger, 1999;54:29–50.
5. Rubio-Viqueira B, Hidalgo M. Direct in vivo xenograft tumor model for predicting chemotherapeutic drug response in cancer patients. Clin Pharmacol Ther 2009;85:217–21.
6. Dong X, Guan J, English JC, et al. Patient-derived first generation xenografts of non-small cell lung cancers: promising tools for predicting drug responses for personalized chemotherapy. Clin Cancer Res 2010;16:1442–51.
7. Hidalgo M, Bruckheimer E, Rajeshkumar NV, et al. A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. Mol Cancer Ther 2011;10:1311–6.
8. Fichtner I, Slisow W, Gill J, et al. Anticancer drug response and expression of molecular markers in early-passage xenotransplanted colon carcinomas. Eur J Cancer 2004;40:298–307.
9. Mischek D, Steinborn R, Petznek H, et al. Molecularly characterised xenograft tumour mouse models: valuable tools for evaluation of new therapeutic strategies for secondary liver cancers. J Biomed Biotechnol 2009;2009:437284.
10. Merk J, Rolff J, Becker M, Leschber G, Fichtner I. Patient-derived xenografts of non-small-cell lung cancer: a pre-clinical model to evaluate adjuvant chemotherapy? Eur J Cardiothorac Surg 2009;36:454–9.
11. Jimeno A, Feldmann G, Suárez-Gauthier A, et al. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Mol Cancer Ther 2009;8:310–4.
12. Russell PJ, Raghavan D, Gregory P, et al. Bladder cancer xenografts: a model of tumor cell heterogeneity. Cancer Res 1986;46:2035–40.
13. Russell PJ, Raghavan D, Philips J, Gregory P. Applications of the xenograft as a model of invasive transitional cell carcinoma of the bladder. Prog Clin Biol Res 1988;260:167–81.
14. Kovnat A, Armitage M, Tannock I. Xenografts of human bladder cancer in immune-deprived mice. Cancer Res 1982;42:3696–703.
15. Kovnat A, Buick RN, Connolly JG, Jewett MA, Keresteci AG, Tannock IF. Comparison of growth of human bladder cancer in tissue culture or as xenografts with clinical and pathological characteristics. Cancer Res 1984;44:2530–3.
16. Zhang H, Aina OH, Lam KS, et al. Identification of a bladder cancer-specific ligand using a combinatorial chemistry approach. Urol Oncol 2012;30:635–45.
17. He X, Marchionni L, Hansel DE, et al. Differentiation of a highly tumorigenic basal cell compartment in urothelial carcinoma. Stem Cells 2009;27:1487–95.
18. Lin TY, Li YP, Zhang H, et al. Tumor-targeting multifunctional micelles for imaging and chemotherapy of advanced bladder cancer. Nanomedicine (Lond) 2012;8:1239–51.
19. Azar HA, Fernandez SB, Bros LM, Sullivan JL. Human tumor xenografts in athymic (nude) mice: chemotherapy trials in serially transplanted tumors. Ann Clin Lab Sci 1982;12:51–9.
20. Matthews PN, Grant AG, Hermon-Taylor J. The growth of human bladder and kidney cancers as xenografts in nude mice and rats. Urol Res 1982;10:293–9.
21. Chan KS, Espinosa I, Chao M, et al. Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. Proc Natl Acad Sci U S A 2009;106:14016–21.
22. Hay JH, Busuttil A, Steel CM, Duncan W. The growth and histological characteristics of a series of human bladder cancer xenografts. Radiother Oncol 1986;7:331–40.
23. Abe T, Tada M, Shinohara N, et al. Establishment and characterization of human urothelial cancer xenografts in severe combined immunodeficient mice. Int J Urol 2006;13:47–57.
24. Hofner T, Macher-Goeppinger S, Klein C, et al. Development and characteristics of preclinical experimental models for the research of rare neuroendocrine bladder cancer. J Urol 2013;190:2263–70.

25. Bernardo C, Costa C, Amaro T, et al. Patient-derived sialyl-Tn-positive invasive bladder cancer xenografts in nude mice: an exploratory model study. Anticancer Res 2014;34:735–44.
26. Sufrin G, McGarry M, Sandberg A, Murphy G. Heterotransplantation of human transitional cell carcinoma in athymic mice. J Urol 1979;121:159–61.
27. Naito S, Iwakawa A, Tanaka K, et al. Heterotransplantation of human urinary bladder cancers in nude mice. Invest Urol 1981;18:285–8.
28. McCue PA, Gomella LG, Veltri RW, Marley GM, Miller MC, Lattime EC. Development of secondary structure, growth characteristics and cytogenetic analysis of human transitional cell carcinoma xenografts in SCID/SCID mice. J Urol 1996;155:1128–32.
29. Park B, Jeong BC, Choi YL, et al. Development and characterization of a bladder cancer xenograft model using patient-derived tumor tissue. Cancer Sci 2013;104:1–8.
30. Johnson JI, Decker S, Zaharevitz D, et al. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. Br J Cancer 2001;84:1424–31.
31. Tentler JJ, Tan AC, Weekes CD, et al. Patient-derived tumour xenografts as models for oncology drug development. Nat Rev Clin Oncol 2012;9:338–50.
32. Peterson JK, Houghton PJ. Integrating pharmacology and in vivo cancer models in preclinical and clinical drug development. Eur J Cancer 2004;40:837–44.
33. Voskoglou-nomikos T, Pater JL, Seymour L. Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models. Clin Cancer Res 2003;9:4227–39.
34. Smith SC, Baras AS, Lee JK, Theodorescu D. The COXEN principle: translating signatures of in vitro chemosensitivity into tools for clinical outcome prediction and drug discovery in cancer. Cancer Res 2010;70:1753–8.

3.3.2 Paper VI

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

Bernardo C, **Costa C**, Amaro T, Gonçalves M, Lopes P, Freitas R, Gärtner F, Amado F, Ferreira JA, Santos L.

Anticancer Res. 2014. Feb; 34(2):735-44.

ANTICANCER RESEARCH *34*: xxx-xxx (2014)

No: 16906-B
Please mark the appropriate section for this paper
☐ Experimental
☐ Clinical
☐ Epidemiological

Patient-derived Sialyl-Tn-positive Invasive Bladder Cancer Xenografts in Nude Mice: An Exploratory Model Study

CARINA BERNARDO[1,9], CÉU COSTA[1,2], TERESINA AMARO[3], MARGARIDA GONÇALVES[1,4], PAULA LOPES[5], RUI FREITAS[6], FÁTIMA GÄRTNER[7,8], FRANCISCO AMADO[9], JOSÉ ALEXANDRE FERREIRA[1,4] and LÚCIO SANTOS[1,2,10]

[1]*Experimental Pathology and Therapeutics Group, Research Center, Portuguese Institute of Oncology Francisco Gentil, Porto, Portugal;*
[2]*Health Faculty, Fernando Pessoa University, Porto, Portugal;*
[3]*Department of Pathology, Hospital Pedro Hispano, Matosinhos, Portugal;*
[4]*Mass Spectrometry Center, QOPNA, Department of Chemistry, University of Aveiro, Aveiro, Portugal;*
[5]*Department of Pathology, Portuguese Institute of Oncology Francisco Gentil, Porto, Portugal;*
[6]*Department of Urology, Portuguese Institute of Oncology Francisco Gentil, Porto, Portugal;*
[7]*Institute of Pathology and Molecular Immunology of the University of Porto (IPATIMUP), Porto, Portugal;*
[8]*Institute of Biomedical Science Abel Salazar, University of Porto (ICBAS-UP), Porto, Portugal;*
9*School of Health Sciences, University of Aveiro (ESSUA), Portugal;*
[10]*Department of Surgical Oncology, Portuguese Institute of Oncology Francisco Gentil, Porto; Portugal*

Abstract. *More than 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. Materials and 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 studied in order to evaluate tumor phenotype maintenance. Results: Tumor take rate was low in the first passage (1/9) but increased and became consistent, therefore suitable for drug testing, in the third passage (13/13). Histology and immunoexpression patterns were similar between primary tumor and xenografts. However, p53 and ki-67 levels increased with passages suggesting a selection of more proliferative clones. STn expression, even though decreased, was preserved in xenografts. Conclusion: We describe the first patient-derived sTn-positive xenograft model to be used for drug testing and identification of prognostic biomarkers.*

Abbreviations: H&E: Hematoxylin and eosin; MIBC: muscle invasive bladder cancer; sTn: sialyl-Tn; FFPE, formalin-fixed, paraffin-embedded.

Correspondence to: Lúcio Lara Santos, Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology Francisco Gentil, Rua Dr. António Bernardino de Almeida 4200-072, Porto, Portugal. Tel: 351 225084000, Fax: 351 225084001, e-mail: llarasantos@gmail.com

Key Words: Human xenografts, urothelial cancer, drug testing, animal models, sialyl-Tn, glycosylation.

Bladder cancer is the fourth most common genitourinary cancer in men and the seventh in women, with an estimated 386,365 new cases and 150,165 deaths yearly (1).

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 (2). The standard treatment for patients with MIBC is radical cystectomy with removal of regional lymph nodes (3). However, up to 50% of patients will relapse with progression to metastatic disease associated with poor survival (4). 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 (3, 5). Previous studies have shown that neoadjuvant platinum-based chemotherapeutics were associated to an absolute risk reduction of 8% in 5-years follow-up (6). However, significant variations in the natural history and response to treatment are seen between MIBC tumors with identical histological features, reflecting a heterogeneity of the constituent tumor cells (7). At the moment, there are no biomarkers available to predict MIBC response to chemotherapy or assist for the design of optimal

treatment schemes, which would translate to better outcomes, reduced toxicity and improved overall survival.

Reflecting the molecular heterogeneity of invasive tumors, we recently reported that approximately 70% of MIBCs 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 (8). The increased expression of ST6GalNac-I leads to STn biosynthesis (9) in several epithelial cancers (*e.g.* gastric, pancreatic, colorectal, ovarian and breast cancers) and is usually associated with poor prognosis (10, 11) Additionally, the sTn antigen contributes to avoidance of metastatic cell elimination in the blood stream by preventing immune recognition (12), modulating the malignant phenotype (13) and enhancing the metastatic ability of cancer cells (14). Therefore, efficient therapies against sTn-positive bladder tumors are warranted; furthermore, and 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 (10) several well-established cancer cell lines of different organs either do not express or lose their ability to present this type of glycosylation (8, 15), denoting a dependence of the tumor microenvironment (8). In an attempt to overcome this limitation bio-engineered cell lines expressing the sTn antigen have been successfully xenografted into mice and were shown responsible for enhancing the metastatic capability of cancer cells (13, 15). 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 (16). However, to our knowledge, the direct xenotransplantation of a sTn-positive bladder tumor into nude mice had not, to this moment, been attempted.

Xenograft models have been used as a 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 (17). 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 the malignant potential of human tumors (18, 19). Patient-derived xenografts mimic the heterogeneity of human cancers and have demonstrated superior correlation of chemosensitivity and specificity data for individualized therapy (17) with prediction rates of 90% and 97% for chemosensitivity and chemoresistance, respectively (20). Previous studies have used cancer xenograft models as a platform for molecular and histopathology studies and therapeutic development with good results in terms of success rates, preservation of the original characteristics of the primary tumor and predictive value of the model (21-24). Preserving the primary tumor characteristics is essential to ensure for 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 (25-27). Still, no evidence has been presented regarding 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 the sTn expression of the primary tumor. Such model is regarded of primary importance to identify for drugs and treatment regimens that would better-serve patients with sTn-positive MIBC. It may also be used to test for novel therapies as well as as a platform to identify markers of tumor response and resistance to drugs.

Materials and Methods

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 1. 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*.

Xenograft establishment. 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.

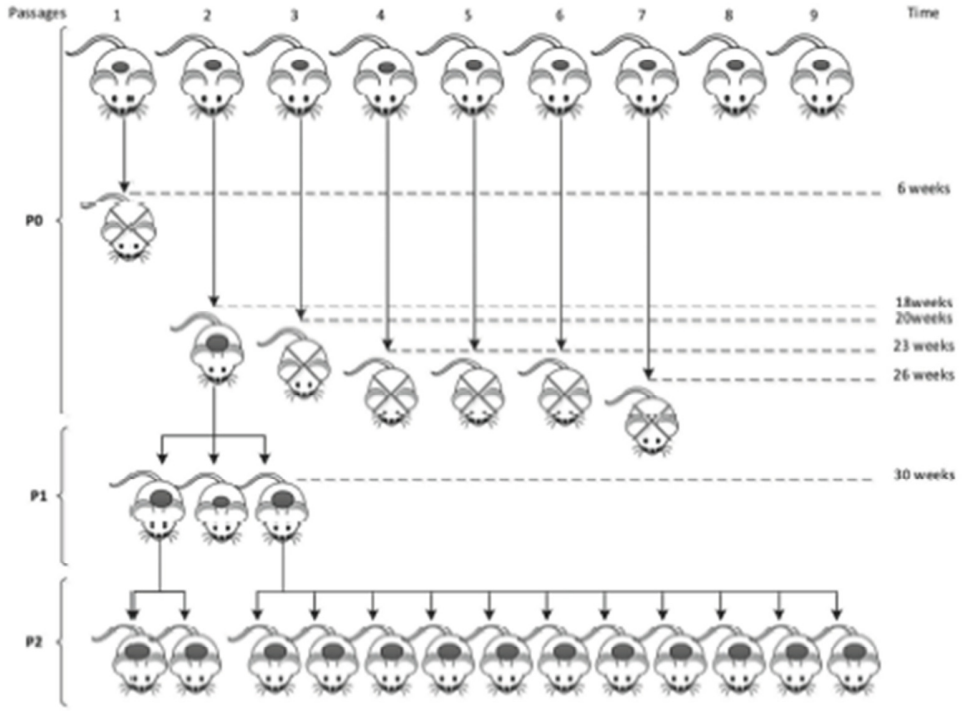

Figure 1. *MIBC tissue was used to establish the xenograft model. After engraftment phase (P0), xenografts were expanded in a cohort of nude mice as described in Materials and Methods. P1 and P2 represent xenografts of the second and third generations, respectively. The red crosses represent sacrificed mice without tumor growth, the small red balls represents the tumors implanted whereas the bigger ones represents tumor growth.*

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 (28).

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:

$$DT = (t2 - t1) ln2 / \ln\left(\frac{V2}{V1}\right)$$

defined by Schwartz (29).

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 histological type, degree of differentiation, nuclear atypia and extension of invasion whereas mice organs were accessed for tumor metastasis.

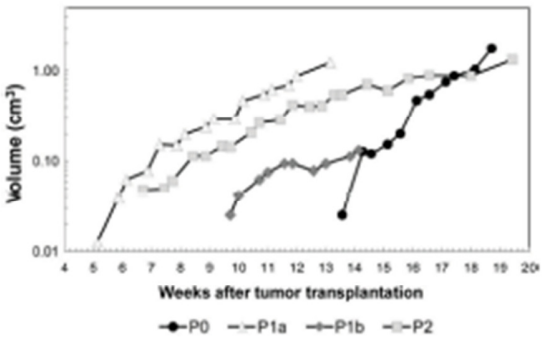

Figure 2. *Tumor growth curve for the first (P0), second P1 (a and b) and third passage (P2) xenografts. In the first passage the xenograft presented a lag period of 13.5 weeks before progressive tumor growth was observable. The time between transplantation and palpable tumor growth became shorter on subsequent passages, 5 (P1a) and 9 (P1b) weeks in the second passage and around 6 weeks in the third. Xenografts growth rate was regular in the three passages. Different lag periods observed for P1 (P1a and P1b) may be due to different amount of initiating tumor cells implanted. Lag periods and tumor growth rates became more constant in the third passage. Tumor volume was plotted on a logarithmic scale.*

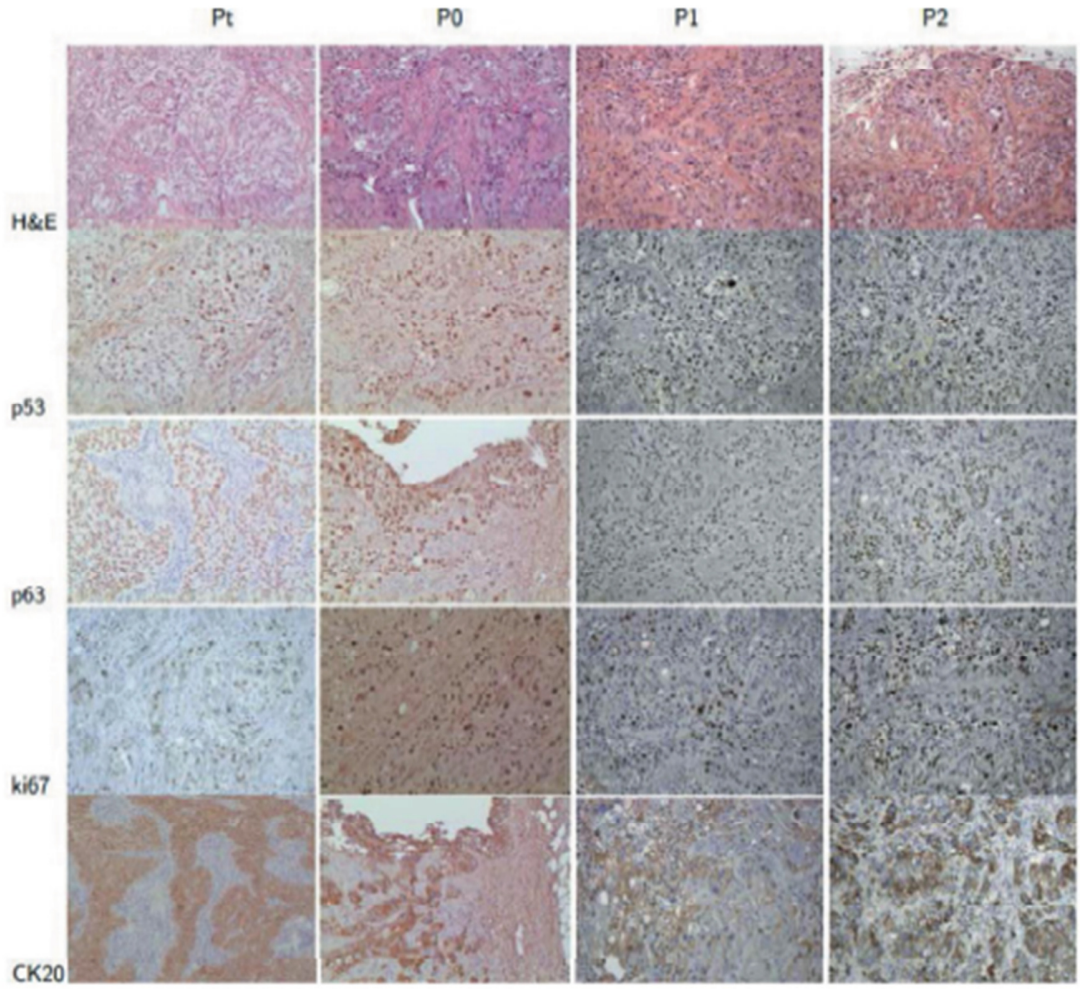

Figure 3. *Histology and tumor molecular markers (p53, p63, ki-67, ck20) immunoexpression of primary tumor (Pt) and first (P0), second (P1), third (P2) generation xenografts (original magnification ×200). No major differences are seen in the tumor structure and cancer cells markers expression between 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.*

Immunohistochemical analysis. Primary tumor and the tumors grown in the three passages were tested for molecular markers expression. Formalin-fixed, paraffin-embedded (FFPE) tumor sections (3 μm) were tested with primary antibodies against p53 (clone DO -7; Dako, Glostrup, Denmark; 1: 200), p63 (clone 4A4; Dako; 1:300), Ki-67 (clone Mlb-1; Dako; 1:150), CK20 (clone Ks20.8; Novocastra Laboratories Ltd., Newcastle, United Kingdom; 1:150) and sTn (anti-sTn TKH2 monoclonal antibody (30); 1:5 from culture supernatant) using polymer-HRP detection method (Power vision, Duivien, The Netherlands). The sections were initially de-waxed in xylene and rehydrated in a graded series of alcohols. Heat-induced epitope retrieval using citrate buffer was carried out according to antibody manufacturer's instructions. Endogenous peroxidase activity was inhibited by immersing sections in 0.6% H_2O_2 in distilled water for 20 min. Sections were rinsed in PBS-Tween prior to incubation with bovine serum albumin solution (20 min) 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 min and DAB substrate for 7 min. Then, the slides were counter-stained with hematoxylin and mounted. Positive and negative controls were run

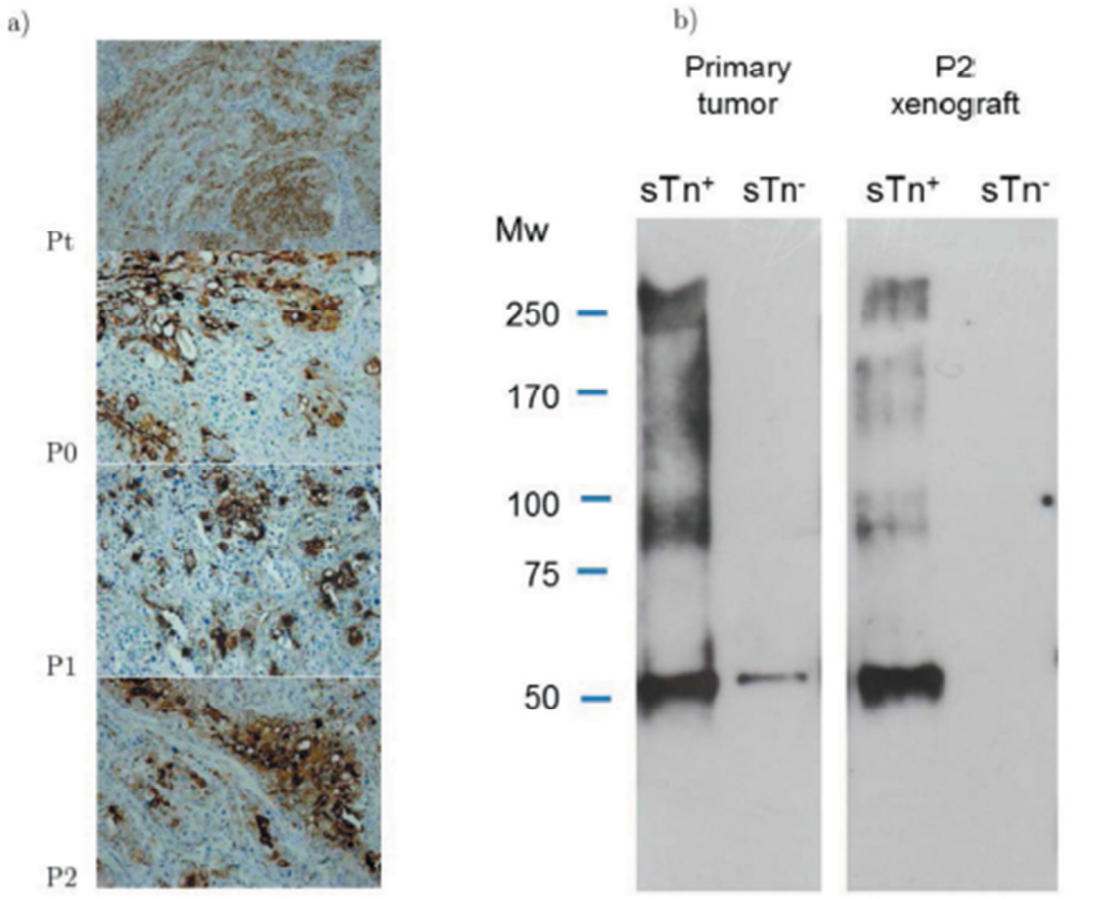

Figure 4. *A) STn immunoexpression in primary tumor and xenografts in first (P0), second (P1) and third generations (P2) (original magnification ×200). STn expression was maintained, even though decreased, in the xenografts. B) Western blot for the proteins expressing the sTn antigen in primary tumor 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.*

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.* (11). 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 microscopy. 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 blotting. Proteins were extracted from FFPE tissues using the Qproteome FFPE tissue kit (Qiagen, Germantown, Maryland, USA). The amount of protein in each extract was estimated with RC protein assay kit (Bio-Rad Laboratories, CA, USA). 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 Uk Limited, Buckinghamshire, UK). Membranes were blocked with 1% carbohydrate depleted carbo-free solution (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at room temperature, incubated overnight at 4°C with anti-sTn TKH2

monoclonal antibody in culture supernatant, washed with TBS-T for 30 min, and finally incubated for 45 h 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 (Bio-Rad). Samples previously desialylated with as previously described by Marcos *et al.* (11) were used as controls.

Results

A direct human bladder cancer xenograft model was established (P0) and expanded (P1) in a cohort of nude mice until the third passage (P2) as schematized in Figure 1.

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). Histological analysis presented a high grade urothelial carcinoma invading the muscularis propria and peri-vesical 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 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 (8).

In the first passage (P0) one out of 9 mice showed 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 2). 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.

Histological and immunohistochemical analysis of the primary tumor and xenografts. The morphological characteristics of xenografs were analyzed and compared to those of 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 histological 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 the 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 3) and the sTn antigen expression (Figure 4), whose overexpression is common in aggressive bladder cancer (31).

Both the primary and the xenografted tumors (P0, P1 and P2) were positive for these markers, reinforcing the homology suggested by histological analysis (Figures 3 and 4). 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 I and Figure 5). 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 5). 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 5). The P0 presented the highest proliferative index, in agreement with the lower tumor doubling time presented by the first generation xenografts (Figure 2). The expression of Ki-67 decreased in P1 to levels similar to those of the initial tumor but increased again in P2. Altogether, these data suggest that the xenograft establishment process may be accompanied by the selection of a more aggressive and proliferative phenotype characterized by a significant overexpression of Ki-67 and p53 (Table I).

The sTn antigen, that was highly expressed in the primary tumor (>75% of the area), was also detected in xenografts (Figure 4A); however its levels decreased with

Table I. *Comparison of immunoexpression for tumor markers p53, p63, Ki-67, CK20 and sTn in primary tumor and third generation xenografts (P2).*

Expression levels	Primary tumor	P2 Xenografts	*p*-Value[a]
p53			
A	1 (100%)	1 (8%)	
B	0	4 (33%)	
C	0	6 (50%)	0.0021
D	0	1 (8%)	
p63			
A	0	0	
B	0	0	
C	0	1 (8%)	0.3173
D	1 (100%)	11 (92%)	
Ki-67			
A	0	0	
B	1 (100%)	6 (50%)	
C	0	6 (50%)	0.0143
D	0	0	
CK20			
A	0	0	
B	0	0	
C	0	1 (%)	0.3173
D	1 (100%)	11 (92%)	
sTn			
A	0	5 (42%)	
B	0	6 (50%)	
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%).

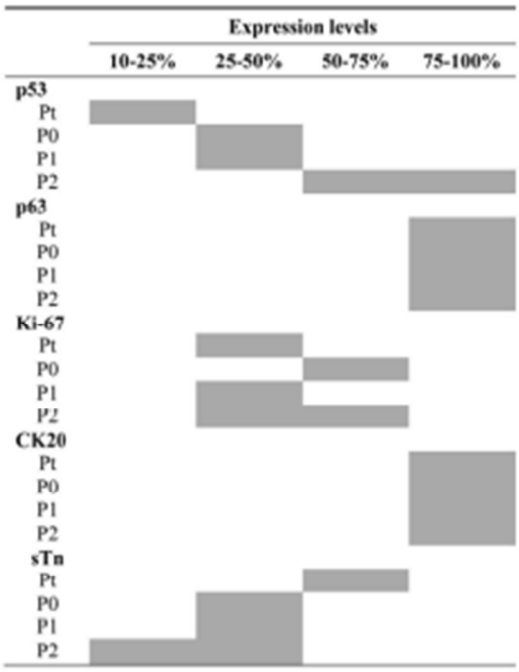

Figure 5. *Immunoexpression of tumor markers p53, p63, Ki-67, CK20 and sTn in the primary tumor (Pt) and first (P0), second (P1) and third (P2) generation xenografts. The expression levels of p63 and CK20 presented by the primary tumor were conserved in the xenografts, indicating similar levels of differentiation. The expression of p53 was increased 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 environment. This proliferation indicator then increased in P2, suggesting the selection of high proliferative clones. The sTn antigen expression was decreased in xenografts when compared with the primary tumor and decreased with the passages.*

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 4B). 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 I).

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 (8). Therefore, the goal of the present study was to establish a direct human bladder cancer xenograft model in nude mice conserving sTn expression of the primary tumor. Such a model is regarded of primary importance to identify drugs and treatment regimens (32) 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 (0.15%) after bilateral transplantation of bladder cancer specimens into 5 immunocompetent mice previously subjected to thymectomy and whole-body radiation (33). Kovnat *et al*., using the same procedure reported a success take rate of 8/33 (0.24%) and 20/53 (0.38%) (34, 35). 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 mm3 per mice (36). 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 (36) as well as with other cancer xenograft models (19, 24, 32, 37).

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 for the most aggressive and rapidly-growing cells from a heterogeneous primary tumor during the 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 (38, 39). 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 pancarcinoma nature of this antigen (10), *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 (24). Altogether we believe that this approach may also be useful in the context of MIBC. Additional work involving a larger patient cohort 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.

Acknowledgements

This work study was supported by the Portuguese Foundation for Science and Technology (FCT) PhD grant SFRH/BD/80855/2011 (CB) and Postdoctoral grant SFRH/BPD/66288/2009 (JAF) and by the "Associação Portuguesa de Urologia". FCT is cofinanced by the European Social Fund (ESF) under Human Potential Operation Programme (POPH) from National Strategic Reference Framework (NSRF). The authors also thank professor Celso A. Reis (IPATIMUP, Portugal) for kindly providing the anti-sTn monoclonal antibody TKH2 used in this study.

References

1 Ferlay J, Shin H-R, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer *127(12)*: 2893-2917, 2010.

2 Sylvester RJ, van der Meijden APM, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, Newling DWW and Kurth K: Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol *49(3)*: 466-477, 2006.

3 Witjes JA, Compérat E, Cowan NC, Santis M De, Gakis G, Lebret T, Ribal MJ and Sherif A: Guidelines on bladder cancer: muscle-invasive and metastatic. Eur Assoc Urol, 2013.

4 Advanced Bladder Cancer (ABC) Meta-analysis Collaboration, C Vale: Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis. Lancet *361(9373)*: 1927-1934, 2003.

5 Bellmunt J, Albiol S, Kataja V; ESMO Guidelines Working Group: Invasive bladder cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. Ann Oncol *20(Suppl 4)*: 79-80, 2009.

6 Sherif A, Holmberg L, Rintala E, Mestad O, Nilsson J, Nilsson S and Malmström P-U: Neoadjuvant cisplatinum based combination chemotherapy in patients with invasive bladder cancer: a combined analysis of two Nordic studies. Eur Urol *45(3)*: 297-303, 2004.

7 Spruck CH, Ohneseit PF, Gonzalez-Zulueta M, Esrig D, Miyao N, Tsai YC, Lerner SP, Schmütte C, Yang S and Cote R: Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res *54(3)*: 784-788,1994.

8 Ferreira JA, Videira P a, Lima L, Pereira S, Silva M, Carrascal M, Severino PF, Fernandes E, Almeida A, Costa C, Vitorino R, Amaro T, Oliveira MJ, Reis CA, Dall'olio F, Amado F and Santos LL: Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol *7(3)*: 719-731, 2013.

9 Marcos NT, Pinho S, Grandela C, Cruz A, Samyn-Petit B, Harduin-Lepers A, Almeida R, Silva F, Morais V, Costa J, Kihlberg J, Clausen H and Reis CA: Role of the human ST6GalNAc-I and ST6GalNAc-II in the synthesis of the cancer-associated sialyl-Tn antigen. Cancer Res *64(19)*: 7050-7057, 2004.

10 Julien S, Videira PA and Delannoy P: Sialyl-Tn in Cancer: (How) Did We Miss the Target? Biomolecules *2(4)*: 435-466, 2012.

11 Marcos NT, Bennett EP, Gomes J, Magalhaes A, Gomes C, David L, Dar I, Jeanneau C, DeFrees S, Krustrup D, Vogel LK, Kure EH, Burchell J, Taylor-Papadimitriou J, Clausen H, Mandel U and Reis CA: ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues. Front Biosci (Elite Ed) *3*: 1443-1455, 2011.

12 Ogata S, Uehara H, Chen A and Itzkowitz SH: Mucin Gene Expression in Colonic Tissues and Cell Lines. Cancer Res *52*: 5971-5978, 1992.

13 Pinho S, Marcos NT, Ferreira B, Carvalho AS, Oliveira MJ, Santos-Silva F, Harduin-Lepers A and Reis CA: Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett *249(2)*: 157-170, 2007.

14 Ozaki H, Matsuzaki H, Ando H, Kaji H, Nakanishi H, Ikehara Y and Narimatsu H: Enhancement of metastatic ability by ectopic expression of ST6GalNAcI on a gastric cancer cell line in a mouse model. Clin Exp Metastasis *29(3)*: 229-238, 2012.

15 Julien S, Krzewinski-Recchi MA, Harduin-Lepers A, Gouyer V, Huet G, Le Bourhis X and Delannoy P: Expression of sialyl-Tn antigen in breast cancer cells transfected with the human CMP-Neu5Ac: GalNAc alpha2,6-sialyltransferase (ST6GalNac I) cDNA. Glycoconj J *18(11-12)*: 883-893, 2003.

16 An Y, Han W, Chen X, Zhao X, Lu D, Feng J and Yang D: A Novel Anti-sTn Monoclonal Antibody 3P9 Inhibits Human Xenografted Colorectal Carcinomas. J Immunother *36(1)*: 20-28, 2012.

17 Pathak AK, Bhutani M, Saintigny P and Mao L: Heterotransplant mouse model cohorts of human malignancies: A novel platform for Systematic Preclinical Efficacy Evaluation of Drugs (SPEED). Am J Transl Res *1(1)*: 16-22, 2009.

18 Johnson JI, Decker S, Zaharevitz D, Rubinstein L V, Venditti JM, Schepartz S, Kalyandrug S, Christian M, Arbuck S, Hollingshead M and Sausville EA: Relationships between drug activity in NCI preclinical *in vitro* and *in vivo* models and early clinical trials. Br J Cancer *84(10)*: 1424-1431, 2001.

19 Rubio-Viqueira B and Hidalgo M: Direct *in vivo* xenograft tumor model for predicting chemotherapeutic drug response in cancer patients. Clin Pharmacol Ther *85(2)*: 217-221, 2009.

20 Fiebig HH, Maier A, and Burger AM: Clonogenic assay with established human tumour xenografts: correlation of *in vitro* to *in vivo* activity as a basis for anticancer drug discovery. Eur J Cancer *40(6)*: 802-820, 2004.

21 Jimeno A, Feldmann G, Suárez-Gauthier A, Rasheed Z, Solomon A, Zou G-M, Rubio-Viqueira B, García-García E, López-Ríos F, Matsui W, Maitra A and Hidalgo M: A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Mol Cancer Ther *8(2)*: 310-314, 2009.

22 Chahinian AP, Mandeli JP, Gluck H, Naim H, Teirstein AS and Holland JF: Effectiveness of cisplatin, paclitaxel, and suramin against human malignant mesothelioma xenografts in athymic nude mice. J Surg Oncol *67(2)*: 104-111, 1998.

23 Russell PJ, Raghavan D, Gregory P, Philips J, Wills EJ, Jelbart M, Wass J, Zbroja RA and Vincent PC: Bladder cancer xenografts: a model of tumor cell heterogeneity. Cancer Res *46(4 Pt 2)*: 2035-2040, 1986.

24 Hidalgo M, Bruckheimer E, Rajeshkumar N V, Garrido-Laguna I, De Oliveira E, Rubio-Viqueira B, Strawn S, Wick MJ, Martell J and Sidransky D: A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. Mol Cancer Ther *10(8)*: 1311-1316, 2011.

25 Serpa J, Mesquita P, Mendes N, Oliveira C, Almeida R, Santos-Silva F, Reis CA, LePendu J and David L: Expression of Lea in gastric cancer cell lines depends on {FUT3} expression regulated by promoter methylation. Cancer Lett *242(2)*: 191-197, 2006.

26 Escrevente C, Machado E, Brito C, Reis CA , Stoeck A, Runz S, Marmé A, Altevogt P and Costa J: Different expression levels of alpha3/4 fucosyltransferases and Lewis determinants in ovarian carcinoma tissues and cell lines. Int J Oncol *29(3)*: 557-566, 2006.

27 Sewell R, Bäckström M, Dalziel M, Gschmeissner S, Karlsson H, Noll T, Gätgens J, Clausen H, Hansson GC, Burchell J and Taylor-Papadimitriou J: The ST6GalNAc-I sialyltransferase

localizes throughout the Golgi and is responsible for the synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer. J Biol Chem *281(6)*: 3586-3594, 2006.

28 Tomayko MM and Reynolds CP: Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol *24(3)*: 148-154, 1989.

29 Schwartz M: A biomathematical approach to clinical tumor growth. Cancer. Wiley Subscription Services, Inc., A Wiley Company *14(6)*: 1272-1294, 1961.

30 Kjeldsen T, Clausen H, Hirohashi S, Ogawa T, Iijima H and Hakomori S: Preparation and Characterization of Monoclonal Antibodies Directed to the Tumor-associated O-linked Sialosyl-2 6 α-N-Acetylgalactosaminyl (Sialosyl-Tn) Epitope. Cancer Res *48(8)*: 2214-2220, 1988.

31 Compérat E, Camparo P, Haus R, Chartier-Kastler E, Bart S, Delcourt A, Houlgatte A, François R, Capron F and Vieillefond A: Immunohistochemical expression of p63, p53 and MIB-1 in urinary bladder carcinoma. A tissue microarray study of 158 cases. Virchows Arch *448(3)*: 319-324, 2006.

32 Tentler JJ, Tan AC, Weekes CD, Jimeno A, Leong S, Pitts TM, Arcaroli, John J, Messersmith WA and Eckhardt SG: Patient-derived tumour xenografts as models for oncology drug development. Nat Rev Clin Oncol *9(6)*: 338-350, 2012.

33 Hay JH, Busuttil A, Steel CM and Duncan W: The growth and histological characteristics of a series of human bladder cancer xenografts. Radiother Oncol *7(4)*: 331-340, 1986.

34 Kovnat A, Buick RN, Connolly JG, Jewett MA, Keresteci AG and Tannock IF: Comparison of Growth of Human Bladder Cancer in Tissue Culture or as Xenografts with Clinical and Pathological Characteristics. Cancer Res *44(6)*: 2530-2553, 1984.

35 Kovnat A, Armitage M and Tannock I: Xenografts of Human Bladder Cancer in Immune-Deprived Mice. Cancer Res *42(9)*: 3696-3703, 1982.

36 Abe T, Tada M, Shinohara N, Okada F, Itoh T, Hamada J-I, Harabayashi T, Chen Q, Moriuchi T and Nonomura K: Establishment and characterization of human urothelial cancer xenografts in severe combined immunodeficient mice. Int J Urol *13(1)*: 47-57, 2006.

37 Ito D, Fujimoto K, Mori T, Kami K, Koizumi M, Toyoda E, Kawaguchi Y and Doi R: *In vivo* antitumor effect of the mTOR inhibitor CCI-779 and gemcitabine in xenograft models of human pancreatic cancer. Int J Cancer *118(9)*: 2337-2343, 2006.

38 Talmadge JE: Clonal Selection of Metastasis within the Life History of a Tumor. Cancer Res *67(24)*: 11471-11475, 2007.

39 Clappier E, Gerby B, Sigaux F, Delord M, Touzri F, Hernandez L, Ballerini P, Baruchel A, Pflumio F and Soulier J: Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse. J Exp Med *208*: 653-661, 2011.

Received November 28, 2013
Revised January 10, 2014
Accepted January 14, 2014

4 Synoptic list of the relevant results

We aim in this chapter to present in a synthetic way the most relevant results observed in the several scientific articles that integrate the PhD thesis.

A. **Evaluation of the expression and PI3K/Akt/mTOR pathway activation and STn in bladder cancer.**

- STn antigen expression was associated with muscle invasion when compared to NMIBC (p=0,001) and correlates with decreased CSS (CSS – cancer specific survival is defined as the period between the tumour removal by surgery and either patient death by cancer or the last follow-up information; log rank p=0,024);
- STn was an independent prognostic marker of worst CSS;
- Tn antigen expression in NMBC and MIBC was residual;
- PI3K/Akt/mTOR pathway related molecules showed an equal and heterogeneous distribution between NMIBC and MIBC and could not be associated with stage of disease and CSS;
- PTEN negative phenotype was significantly associated with muscle invasion;
- PTEN negative tumours had lower CSS (p=0,015);
- Overexpression of pAKT, pmTOR and/or pS6 allowed discriminating STn-positive advanced stage bladder tumours facing worst CSS (p=0,027);
- Overexpression of PI3K/AKT/mTOR pathway proteins in STn+ MIBC was independently associated with risk of death by cancer: 6-fold higher, p=0,039);
- STn is a marker of poor prognosis in bladder cancer. Its combination with PI3K/AKT/mTOR pathway evaluation has potential to be used in disease stage stratification;
- Several MIBC presented an increased pS6 staining intensity in the invasion front.

To use murine chemically induced urothelial tumours treated with Sirolimus to assess the impact of mTOR inhibitors on cell protein expression

- Mice treated with sirolimus presented decreased number of invasive lesions and concomitantly decreased expression of pS6 and STn;

- Mice experiments suggested that mTOR pathway inhibition with sirolimus has therapeutic potential in MIBC.

To evaluate the regulation of PI3K/Akt/mTOR and activation of the ubiquin- proteosome pathways in bladder cancer- induced skeletal muscle wasting

- The histologic evidences of non-muscle-invasive bladder tumours observed in BBN animals were related to 17% loss of body weight- caquexia;
- High serum levels of pro-inflammatory mediators and myostatin and high levels of urinary MMPs;
- Decreased oxidative metabolism in wasted muscle;
- Reduced levels of pAKT, mTOR and P70S6K were observed;
- It was observed activation of the ubiquitin-proteasome protein degradation pathway and suppression of PI3K/AKT/mTOR pathway.

Expression of STn in bladder tumours its correlation with tumour proliferation and clinical outcome

- 75% of the high-grade bladder tumours, presenting elevated proliferation rates and high risk of recurrence/progression expressed STn, mainly in non-proliferative areas of the tumour - in cells invading the basal and muscle layers;
- STn was found in tumour-adjacent mucosa but was not expressed by the normal urothelium;
- STn expression correlates with the concomitant increase of cell motility and invasive capability.

B. <u>Everolimus (RAD001) combination with Cisplatin as a potential strategy to achieve high levels of efficacy with lower cytotoxicity</u>

- Treatment with cisplatin statistically significantly ($P<0.05$) decreased cell proliferation in cell lines in a dose-dependent manner : cell- cycle arrest in the late S/G2 phase followed by a higher apoptotic rate;

- Combination of everolimus and cisplatin or gemcitabine decreased the proliferation of bladder cancer cell lines -statistically significant ($P<0.05$) in comparison to the chemotherapy agent alone;
- The NMI cancer cell line was the most sensitive to the action of the combination everolimus/cisplatin.

C. <u>Direct bladder cancer xenografts model</u>

- It was stablished tumour xenografts with preservation of the original tumour identity but variable take rate;
- Advanced stage and high grade tumours were associated with increased take rate;
- Histology and immunoexpression of tumour markers (p53, p63, Ki-67, CK20, STn) were similar between primary tumours and xenografts;
- p53 and ki-67 levels of expression increased;
- STn expression, even though decreased, was preserved in xenografts - STn-positive xenograft model.

5 Final Discussion and Conclusions

Bladder cancer is the second most common cancer of the urinary tract [Willems and Stein, 2004] and in Portugal according to Roreno was one of the malignant diseases more frequent in man resulting in 721 deaths [Roreno, 2008 and Ferlay *et al*, 2010].

Bladder cancer is a heterogeneous disease whether in pathology or in the response to therapeutics urging to develop new strategies of research and selecting the best treatments [Kaufman *et al*, 2009].

NMIBC represents about 75% of the newly diagnosed cases and are characterized by a high recurrence rate [Holmang *et al*, 1995]. MIBC represents 20-30% of the cases and treatment includes cystectomy and cisplatin-based chemotherapy regime. Fifty percent of this patient will relapse and progress to metastatic disease. For both groups we need to develop new drugs with higher efficacy and lower cytotoxicity [Bernardo *et al*, 2014].

The PI3K/AKT/mTOR is an important signaling cascade determinant for cell growth and proliferation and disruption along this pathway can result in altered cell dynamics and tumour development.

mTOR pathway alterations have been shown to occur in bladder cancer, such as PIK3CA gene mutations, elevations of pAKT levels and loss of TSC1 function [Ching and Hansel, 2010]. The aberrant activation of this pathway has been suggested to contribute to invasion [Wu *et al*, 2004]. Previous studies also demonstrated that STn overexpression is a key player in a subset of advanced stage tumours making the STn antigen a potential anti-cancer target [Ferreira *et al*, 2013].

In the group of scientific articles presented in this thesis we intended to evaluate the expression of PI3K/AKT/mTOR activation in bladder cancer both in humans and in a chemical induced murine model. We found that PI3K/AKT/mTOR pathway related molecules show an equal distribution between NMIBC and MIBC and did not associated with stage of disease. Thus activation of mTOR pathway proteins did not discriminate the stage of disease or a worse prognosis reinforcing previous observations [Korkolopoulou *et al*, 2012; Fahmy *et al* 2013]. In contrast we found that PTEN expression was decrease in advanced stage tumours as previously reported [Saal *et al*, 2007]. We also observed that PTEN negative MIBC was significantly

associated with muscle invasion and lower CSS. According with previous observations PTEN expression exerts a suppressive effect over the PI3K/AKT/mTOR pathway which may account for poorer outcome [Sun *et al*, 2011; Calderaro *et al*, 2014].

STn expression is associated with muscle invasive and correlate with decrease survival as previously observed in different cancer models such as gastric tumours [Tsuchiya *et al*, 1995]. In bladder cancer is expressed mainly in advanced stages cases [Ferreira *et al*, 2013]. It seems that STn expression altered cancer cells properties being a key event leading to metastasis also observed by Lin and colleagues [Lin *et al*, 2009]. Overexpression of PI3K/AKT/mTOR pathway biomarkers were associated with worse CSS in STn positive advanced stage tumours.

These results suggest that these aggressive tumours may be good candidate to mTOR inhibitors therapeutics combining drugs targeting STn expressing cells, although these preliminary results were obtained from a relatively low number of patients.

Regarding PTEN, we observed that PTEN negative MIBC presented worse CSS in comparison to PTEN positive lesions. PTEN expression was decreased in advanced stage tumours as previously reported [Sun *et al*, 2011; Calderaro *et al*, 2014].

Using murine chemically induced urothelial tumours we assessed the impact of mTOR inhibitors on cell protein expression, with sirolimus. Mice treated with sirolimus presented a decrease number of invasive tumours and concomitantly decreased expression of pS6 and STn, as supported by Oliveira and colleagues 2009 [Oliveira *et al*, 2009]. Using this model we reinforce that sirolimus reduce the frequency of tumours with invasive potential and significantly decreased the expression of pS6, one of the downstream effectors of the PI3K/AKT/mTOR. The administration of sirolimus was effective against STn positive cells – we describe for the first time that chemically-induced bladder tumours express this antigen in contrast with most of the available bladder cancer cell lines. These observations also demonstrate for the first time an association between PI3K/AKT/mTOR and STn in invasive tumours (STn antigen is biomarker of poor prognosis). This also provides the first link between two related events in bladder cancer - PI3K/AKT/mTOR activation and altered glycosylation. More studies need to be done before progressing to clinical trials.

The role of the mTOR inhibitors was also evaluated using bladder cancer cell lines. Regarding this topic and the combination of everolimus with cisplatin as potential strategy to use in bladder cancer achieving high levels of efficacy with lower cytotoxicity we published the following results: treatment with cisplatin statistically decreased cell proliferation in cell lines in dose dependent manner ($p<0.05$). Cell cycle arrested in the late S/G2 phase followed by a high apoptotic rate. Combination therapy was already performed previously in some preclinical studies namely on advanced solid tumours [Fury *et al*, 2012]. Combination of everolimus and cisplatin decreased the proliferation of bladder cancer cell lines in comparison to the chemotherapy agent alone ($p<0,05$). The anti-proliferative activity of cisplatin combined with everolimus could be a useful anticancer treatment for patients with bladder cancer. We also observed that the NMIBC cell line was the most sensitive to the action of combined cisplatin/everolimus, thus opening a new line of research in this group of bladder tumours.

To evaluate the correlation between the expression of PI3K/AKT/mTOR pathway activation in bladder cancer-induced skeletal muscle wasting we found that in rat model BBN induced urothelial carcinoma there was a 17% of body weight loss - cachexia. These tumours were equivalent to the NMIBC observed in humans, a well-stablished model of tumours of the urinary tract [Palmeira *et al*, 2009]. It was also observed high serum levels of pro-inflammatory mediators, myostatin, high levels of urinary MMPs and decreased oxidative metabolism in wasted muscle supporting a catabolic profile associated with carcinoma-induced muscle wasting. We found reduce levels of pAKT, mTOR and P70S6K suggesting unbalance between protein synthesis and degradation as previously suggested [Fanzani *et al*, 2012]. We observed activation of the ubiquitin-proteossome protein degradation pathway and suppression of PI3K/AKT/mTOR pathway. A therapeutic measure that prevents/counteract this imbalance might limit comorbid disease and consequently enhance patients' quality of life.

Regarding the expression of STn in bladder carcinomas and its correlation with tumour proliferation and clinical relevance we found that 75% of the high grade bladder tumours, expressing STn presented elevated proliferation rates and high risk of recurrence/progression, as previously observed by Davidson and colleagues [Davidson *et al*, 2000]. This 75% was found

mainly in non-proliferative areas of the tumour, in cells invading the basal and muscle layers. Thus STn was correlated with the depth of invasion and metastization [Ikeda *et al*, 1993].

STn expression seems to correlate with the concomitant increase of cell motility and invasive capability but despite this observation there is still little information regarding STn expression in bladder cancer. Furthermore it was not express by the normal urothelium demonstrating the cancer-specific nature of this antigen.

As referred before more studies need to be done before progressing to clinical trials. Human tumour xenograft models have been developed for several types of cancer however in the case of human bladder cancer few studies are available [Bernardo *et al*, 2015].

We established a xenograft model of bladder cancer suitable for comparison between tumour response in mice and clinical outcome in human bladder tumours. In the group of scientific articles that resulted from this work we studied the best methodology to develop a tumour xenograft model of bladder cancer preserving the original tumour identity. Although previous studies suggested a correlation between engraftment success and clinical prognosis there is still few evidence of this association [Bellmunt *et al*, 2009]. We observed advanced stage and high grade tumours were associated with increase take rate. Mores studies need to be done before stablish the model suitable for drugs testing.

Histology and imunoexpression of tumour markers (p53, p63, KI-67, CK20, STn) were similar between primary tumours and xenograft. However p53 and Ki-67 levels of expression increased suggesting a tendency for the most aggressive and rapidly growing cells from a heterogeneous primary tumour during the engraftment process as observed before [Talmadge, 2007]. STn expression even though decreased was preserved in xenografts reinforcing a STn positive xenograft model. Given the pancarcinoma nature of STn antigen expression, its association with invasion and metastases, these findings may constitute valuable insights for other tumours.

The published results included in the present thesis show that the activation of PI3K/AKT/mTOR pathway is present either in NMIBC and MIBC, suggesting a key role in bladder cancer carcinogenesis. Moreover chemosensibility was observed in both bladder cancer groups when

mTOR inhibitors were used. These results suggested that the combination of these molecules with the established therapeutic drugs may be potential strategy to use in bladder cancer achieving high levels of efficacy with lower cytotoxicity. To achieve this goal the new experimental bladder cancer models, namely xenografts models, are very important, allowing the study of these new molecular targets and evaluate the tumour response to these new drugs or drug combinations in the field of urologic oncology.

6 Future perspectives

The series of studies presented in this PhD thesis have the purpose to identify more effective therapeutic targets for bladder cancer due to its recurrence and potential to progress to invasion, especially the ones with poor prognosis. Alterations in the activation of the PI3K/Akt/mTOR pathway are key events in bladder cancer development. New studies that allow a better understanding of the upstream and downstream mTOR pathway regulation should be performed.

We also addressed the expression of STn, a cancer associated antigen and its possible association with active PI3K/Akt/mTOR in invasive tumors. Tumours presenting positive pmTOR and/or pS6 showed a trend towards decrease survival in MIBC STn positive. Such results suggest that these tumours may be good candidate to mTOR inhibitors therapeutics such as sirolimus combined therapeutics against STn-expressing cells. These results should be confirmed with a large series of bladder cancer patients and other mTOR inhibitors may be assayed.

As a complement to this work we studied patient- derived tumor xenografts and its possible use to compare tumor response and resistance to drugs, in bladder cancer. We established a xenograft model that preserved the original tumour identity and could be used for comparison between tumour response in mice and clinical outcome in human bladder tumours. We also access the potential of combined Everolimus with Cisplatin in bladder cancer cell lines as a useful anti-cancer drug therapy.

In the future, using standard animal models, xenografts or cell lines we may access the importance of the inhibitors of the PI3K/Akt/mTOR pathway and use it for drug testing and identification of prognostic biomarkers; also further studies regarding PI3K/Akt/mTOR pathway abnormalities need to be addressed for patient stratification and personalized therapies not only for bladder but also for other types of cancer.

7 References

Afonso J, Longatto-Filho A, DA Silva VM, Amaro T, Santos LL (2014) Phospho-mTOR in non-tumour and tumour bladder urothelium: Pattern of expression and impact on urothelial bladder cancer patients. Oncol Lett, 8(4):1447-1454.

Alberg AJ, Kouzis A, Genkinger JM, Gallicchio L, Burke AE, Hoffman SC, Diener-West M, Helzlsouer KJ, Comstock GW (2007) A prospective cohort study of bladder cancer risk in relation to active cigarette smoking and house hold exposure to secondhand cigarette smoke. Am J Epidemiol, 165:660-666.

Amato RJ, Flaherty A, Zhang Y, Ouyang F, Mohlere V (2013) Clinical prognostic factors associated with outcome in patients with renal cell cancer with prior tyrosine kinase inhibitors or immunotherapy treated with everolimus. Urol Oncol, 32(3):345-54.

Arentsen HC, Hendricksen K, Oosterwijk E, Witjes JA (2009) Experimental rat bladder urothelial cell carcinoma models. World J Urol, 27: 313-317.

Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BW, Compérat E, Sylvester RJ, Kaasinen E, Böhl A, Palou Redorta J, Rouprêt M (2013) EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update. Eur Urol, 64:639-53.

Becci PJ, Thompson HJ, Strum JM, Brown CC, Sporn MB, Moon RC (1981) N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder cancer in C57BL/6 X DBA/2 F1 mice as a useful model for study of chemoprevention of cancer with retinoids. Cancer Res, 41: 927-932.

oisBecker MN, Wu KJ, Marlow LA, Kreinest PA, Vonroemeling CA, Copland JA, Grewe M, Gansauge F, Schmid RM, Adler G, Seufferlein T (1999) Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6K pathway in human pancreatic cancer cells. Cancer Res, 59: 3581-3587.

Bellmunt J (2011) What are the expected developments in the medical treatment of bladder cancer. Eur J Cancer, 47 Suppl 3:S294-7.

Bellmunt J, Albiol S, Kataja V (2009) Invasive bladder cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. ESMO Guidelines Working Group. Ann Oncol, 20 Suppl 4:79-80.

Bernardo C, Costa C, Amaro T, Gonçalves M, Lopes P, Freitas R, Gärtner F, Amado F, Ferreira JA, Santos L (2014) Patient-derived sialyl-Tn-positive invasive bladder cancer xenografts in nude mice: an exploratory model study. Anticancer Res, 34(2):735-44.

Bernardo C, Costa C, Sousa N, Amado F, Santos L (2015) Patient-derived bladder cancer xenografts: a systematic review. Transl Res, 12. pii: S1931-5244 (15)00040-7.

Bjornsti MA, Houghton P (2004) The TOR pathway: a target for cancer therapy. Nat Rev Cancer, 4:335-348.

Bjornsti MA, Houghton PJ (2004) The TOR pathway: a target for cancer therapy. Nat Rev Cancer, 4:335-348.

Bonetto A, Aydogdu T, Kunzevitzky N, Guttridge DC, Khuri S, Koniaris LG, Zimmers TA (2011) STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia. PLoS One, 6: e22538.

Bose S, Chandran S, Mirocha JM, Bose N. (2006) The Akt pathway in human breast cancer: a tissue-array-based analysis. Mod Pathol, 19(2):238-45.

Brockhausen I (1999) Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta, 6; 1473(1):67-95.

Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, Kassouf W, Kiemeney LA, La Vecchia C, Shariat S, Lotan Y (2013) Epidemiology and risk factors of urothelial bladder cancer. Eur Urol, 63:234-241.

Buskermolen S, Langius JA, Kruizenga HM, Ligthart-Melis GC, Heymans MW, Verheul HM (2012) Weight loss of 5% or more predicts loss of fat-free mass during palliative chemotherapy in patients with advanced cancer: a pilot study. Nutr Cancer, 64:826-832.

Cafferkey R, Young PR, McLaughlin MM, Bergsma DJ, Koltin Y, Sathe GM, Faucette L, Eng WK, Johnson RK, Livi GP (1993) Dominant missense mutations in a novel yeast protein related to

mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. Mol Cell Biol, 13:6012-6023.

Calderaro J, Rebouissou S, de Koning L, Masmoudi A, Hérault A, Dubois T, Maille P, Soyeux P, Sibony M, de la Taille A, Vordos D, Lebret T, Radvanyi F, Allory Y (2014) PI3K/AKT pathway activation in bladder carcinogenesis. Int J Cancer, 134:1776-84.

Cao Y, Stosiek P, Springer GF, Karsten U (1996) Thomsen-Friedenreich-related carbohydrate antigens in normal adult human tissues: a systematic and comparative study. Histochem Cell Biol, 106 (2):197-207.

Carracedo A, Baselga J, Pandolfi PP (2008) Deconstructing feedback-signaling networks to improve anticancer therapy with mTORC1 inhibitors. Cell Cycle, 15(24):3805-9.

Carrascal MA, Severino PF, Guadalupe Cabral M, Silva M, Ferreira JA, Calais F, Quinto H, Pen C, Ligeiro D, Santos LL, Dall'Olio F, Videira PA (2014) Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. Mol Oncol, 8:753-65.

Charlton ME, Adam MP, Sun L, Deorah S (2014) Bladder cancer collaborative stage variables and their data quality, usage, and clinical implications: a review of SEER data, 2004-2010. Cancer, 120 Suppl 23:3815-3825.

Chaux A, Compérat E, Varinot J, Hicks J, Lecksell K, Solus J, Netto GJ (2013) High levels of phosphatase and tensin homolog expression are associated with tumor progression, tumor recurrence, and systemic metastases in pT1 urothelial carcinoma of the bladder: a tissue microarray study of 156 patients treated by transurethral resection. Urology, 81(1):116-22.

Chen M, Gu J, Delclos GL, Killary AM, Fan Z, Hildebrandt MA, Chamberlain RM, Grossman HB, Dinney CP, Wu X (2010) Genetic variations of the PI3K-AKT-mTOR pathway and clinical outcome in muscle invasive and metastatic bladder cancer patients. Carcinogenesis, 31:1387-1391.

Cheng H, Walls M, Baxi SM, Yin MJ (2013) Targeting the motor pathway in tumor malignancy. Curr Cancer Drug Targets, 13(3):267-77.

Cheung G, Sahai A, Billia M, Dasgupta P, Khan MS (2013) Recent advances in the diagnosis and treatment of bladder cancer. BMC Med, 11:13.

Ching CB, Hansel DE (2010) Expanding therapeutic targets in bladder cancer: the PI3K/Akt/mTOR pathway. Lab Invest, 90(10):1406-14.

Chiong E, Esuvaranathan K (2010) New therapies for non-muscle-invasive bladder cancer. World J Urol, 28:71–78.

Clément M, Rocher J, Loirand G, Le Pendu J (2004) Expression of sialyl-Tn epitopes on beta1 integrin alters epithelial cell phenotype, proliferation and haptotaxis. J Cell Sci, 1; 117 (Pt 21): 5059-69.

Colombel M, Soloway M, Akaza H, Bohle A, Palou J, Buckley R, Lammg D, Brausi M, Witjes JA, Persad R (2008) Epidemiology, Staging, Grading, and Risk Stratification of Bladder Cancer. Eur Urol Suppl, 7:618-626

Cross W, Whelan P (2010) Bladder Cancer. Surgery, 28 (12):599-604.

Dall'Era MA, Cheng L, Pan CX (2012) Contemporary management of muscle-invasive bladder cancer. Expert Rev Anticancer Ther, 12:941-50.

Davidson B, Berner A, Nesland JM, Risberg B, Kristensen GB, Tropé CG, Bryne M (2000) Carbohydrate antigen expression in primary tumors, metastatic lesions, and serous effusions from patients diagnosed with epithelial ovarian carcinoma: evidence of up-regulated Tn and Sialyl Tn antigen expression in effusions. Hum Pathol, 31(9):1081-7.

Dong X, Guan J, English JC, Flint J, Yee J, Evans K, Murray N, Macaulay C, Ng RT, Gout PW, Lam WL, Laskin J, Ling V, Lam S, Wang Y (2010) Patient-derived first generation xenografts of non-small cell lung cancers: promising tools for predicting drug responses for personalized chemotherapy. Clin Cancer Res, 1; 16(5):1442-51.

Dutcher JP (2004) Mammalian target of rapamycin inhibition. Clin Cancer Res, 10(18 Pt 2):6382S-7S.

Edge SB (2010) American Joint Committee on Cancer, American Cancer Society. AJCC cancer staging handbook: from the AJCC cancer staging manual, 7th New York: Springer.

Efeyan A, Zoncu R, Sabatini DM (2012) Amino acids and mTORC1: from lysosomes to disease. Trends Mol Med, 18:524-33.

Fahmy M[1], Mansure JJ, Brimo F, Yafi FA, Segal R, Althunayan A, Hicks J, Meeker A, Netto G, Kassouf W (2013) Relevance of the mammalian target of rapamycin pathway in the prognosis of patients with high-risk non-muscle invasive bladder cancer. Hum Pathol, 44(9):1766-72.

Fajkovic H, Halpern JA, Cha EK, Bahadori A, Chromecki TF, Karakiewicz PI, Breinl E, Merseburger AS, Shariat SF (2011) Impact of gender on bladder cancer incidence, staging, and prognosis. World J Urol, 29:457-463.

Fantus D, Thomson AW (2015) Evolving Perspectives of mTOR Complexes in Immunity and Transplantation. Am J Transplant, 15:891-902.

Fanzani A, Conraads VM, Penna F, Martinet W (2012) Molecular and cellular mechanisms of skeletal muscle atrophy: an update. J Cachexia Sarcopenia, 3(3):163-79.

Fasolo A, Sessa C (2012) Targeting mTOR pathways in human malignancies. Curr Pharm Des, 18:2766-2777.

Ferlay J, S.H., Bray F, Forman D, Mathers C, Parkin DM (2010) GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10 2010, International Agency for Research on Cancer: Lyon, France.

Ferlay J, Shin HR, Bray F, Formab D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer, 2010. 127(12): p. 2893-917.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2013) GLOBOCAN 2012 v 1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11. Lyon, France: International Agency for Research on Cancer.

Ferreira JA, Videira PA, Lima L, Pereira S, Silva M, Carrascal M, Severino PF, Fernandes E, Almeida A, Costa C, Vitorino R, Amaro T, Oliveira MJ, Reis CA, Dall'Olio F, Amado F, Santos LL (2013) Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol, 7:719-731.

Fichtner I, Slisow W, Gill J, Becker M, Elbe B, Hillebrand T, Bibby M (2004) Anticancer drug response and expression of molecular markers in early-passage xenotransplanted colon carcinomas. Eur J Cancer, 40(2):298-307.

Fury MG, Sherman E, Ho A, Katabi N, Sima C, Kelly KW, Nwankwo O, Haque S, Pfister DG (2012) A phase I study of temsirolimus plus carboplatin plus paclitaxel for patients with recurrent or metastatic (R/M) head and neck squamous cell cancer (HNSCC). Cancer Chemother Pharmacol, 70(1):121-8.

Gallangher DJ, Milowsky MI, Bajorin DF (2008) Advanced bladder cancer: status of first-line chemotherapy and the search for active agents in the second-line setting. Cancer, 113:1284-93

Gerullis H, Eimer C, Ecke TH, Georgas E, Freitas C, Kastenholz S, Arndt C, Heusch C, Otto T (2012) A phase II trial of temsirolimus in second-line metastatic urothelial cancer. Med Oncol, 29:2870-2876.

Gillet JP, Varma S, Gottesman MM (2013) The clinical relevance of cancer cell lines. J Natl Cancer Inst, 3; 105(7):452-8.

Greene FL (2002) American Joint Committee on Cancer. American Cancer Society. AJCC cancer staging manual, 6th New York: Springer-Verlag.

Grewe M, Gansauge F, Schmid RM, Adler G, Seufferlein T (1999) Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6K pathway in human pancreatic cancer cells. Cancer Res, 59: 3581-3587.

Griffiths T (2013) Current perspectives in bladder cancer management. Int J Clin Pract, 65:435–448.

Griffiths TR (2012) Action on Bladder Cancer. Current perspectives in bladder cancer management. Int J Clin Pract, 67:435-448.

Guancial EA, Rosenberg JE (2015) The role of genomics in the management of advanced bladder cancer. Curr Treat Options Oncol, 16:319.

Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer. Cancer Cell, 12:9-22.

Hansel DE, Platt E, Orloff M, Harwalker J, Sethu S, Hicks JL, De Marzo A, Steinle RE, Hsi ED, Theodorescu D, Ching CB, Eng C (2010) Mammalian target of rapamycin (mTOR) regulates cellular proliferation and tumor growth in urothelial carcinoma. Am J Pathol, 176:3062-72.

Harling M, Schablon A, Schedlbauer G, Dulon M, Nienhaus A (2010) Bladder Cancer among hairdressers: a meta-analysis. Occup Environ Med, 67:351-358.

Hartmann A, Schlake G, Zaak D, Hungerhuber E, Hofstetter A, Hofstaedter F, Knuechel R. (2002) Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma in situ of human urinary bladder. Cancer Res, 62: 809-818.

Hay N (2005) The Akt-mTOR tango and its relevance to cancer. Cancer Cell, 8(3):179-83.

Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev, 18:1926-1945.

Hidalgo M, Bruckheimer E, Rajeshkumar NV, Garrido-Laguna I, De Oliveira E, Rubio-Viqueira B, Strawn S, Wick MJ, Martell J, Sidransky D (2011) A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. Mol Cancer Ther, 10(8):1311-6.

Hodges KB, Lopez-Beltran A, Davidson DD, Montironi R, Cheng L (2010) Urothelial dysplasia and other flat lesions of the urinary bladder: clinicopathologic and molecular features. Hum Pathol, 41:155-162.

Holmäng S, Hedelin H, Anderström C, Johansson SL. (1995) The relationship among multiple recurrences, progression and prognosis of patients with stages Ta and T1 transitional cell cancer of the bladder followed for at least 20 years. J Urol, 153(6):1823-6.

Huang K, Fingar DC (2014) Growing knowledge of the mTOR signaling network. Semin Cell Dev Biol, 36:79-90.

Huang S, Houghton PJ (2001) Resistance to rapamycin: a novel anticancer drug. Cancer Metastasis Rev, 20: 69-78.

Ikeda Y, Kuwano H, Baba K, Ikebe M, Matushima T, Adachi Y, Mori M, Sugimachi K (1993) Expression of Sialyl-Tn antigens in normal squamous epithelium, dysplasia, and squamous cell carcinoma in the esophagus. Cancer Res, 53 (7):1706-8.

Inoki K, Ouyang H, Li Y, Guan KL (2005) Signaling by target of rapamycin proteins in cell growth control. Microbiol Mol Biol Rev, 69(1):79-100.

Jacobs BL, Lee CT, Montie JE (2010) Bladder cancer in 2010: how far have we come? CA Cancer J Clin, 60:244–72.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin, 61:69-90.

Johns N, Hatakeyama S, Stephens NA, Degen M, Degen S, Frieauff W, Lambert C, Ross JA, Roubenoff R, Glass DJ, Jacobi C, Fearon KC (2014) Clinical classification of cancer cachexia: phenotypic correlates in human skeletal muscle. PLoS One, 9(1):e83618.

Johnson JI, Decker S, Zaharevitz D, Rubinstein LV, Venditti JM, Schepartz S, Kalyandrug S, Christian M, Arbuck S, Hollingshead M, Sausville EA (2001) Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. Br J Cancer, 84:1424-31.

Julien S, Krzewinski-Recchi MA, Harduin-Lepers A, Gouyer V, Huet G, Le Bourhis X, Delannoy P (2001) Expression of sialyl-Tn antigen in breast cancer cells transfected with the human CMP-Neu5Ac: GalNAc alpha2,6-sialyltransferase (ST6GalNac I) cDNA. Glycoconj, 18(11-12):883-93.

Julien S, Adriaenssens E, Ottenberg K, Furlan A, Courtand G, Vercoutter-Edouart AS, Hanisch FG, Delannoy P, Le Bourhis X. (2006) ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology, 16(1):54-64.

Julien S, Videira PA, Delannoy P (2012) Sialyl-Tn in cancer: (how) did we miss the target? Biomolecules, 2(4):435-66.

Kaufman DS, Shipley WU, Feldman AS (2009) Bladder cancer. Lancet, 374(9685):239-249.

Kirkali Z, Chan T, Manoharan M, Algaba F, Busch C, Cheng L, Kiemeney L, Kriegmair M, Montironi R, Murphy WM, Sesterhenn IA, Tachibana M, Weider J (2005) Bladder cancer: epidemiology, staging and grading, and diagnosis. Urology, 66(6 Suppl 1):4-34.

Klotz L Brausi M (2015) World Urologic Oncology Federation Bladder Cancer Prevention. Urol Oncol, 33:25–29.

Knowles MA (2008) Molecular pathogenesis of bladder cancer. Int J Clin Oncol, 13:287-297.

Knowles MA, Hurst CD (2015) Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer, 15:25-41.

Kompier LC, Lurkin I, van der Aa MN, van Rhijn BW, van der Kwast TH, Zwarthoff EC (2010) FGFR3, HRAS, KRAS, NRAS and PIK3CA mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. PLoS One, 3;5(11):e13821.

Korkolopoulou P, Levidou G, Trigka EA, Prekete N, Karlou M, Thymara I, Sakellariou S, Fragkou P, Isaiadis D, Pavlopoulos P, Patsouris E, Saetta AA (2012) A comprehensive immunohistochemical and molecular approach to the PI3K/AKT/mTOR (phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene/mammalian target of rapamycin) pathway in bladder urothelial carcinoma. BJU Int, 110(11 Pt C):E1237-48.

Kulkarni GS, Finelli A, Fleshner NE, Jewett MA, Lopushinsky SR, Alibhai SM (2007) Optimal management of high-risk T1G3 bladder cancer: a decision analysis. PLoS Med, 4:e284.

Lamb BW, Vasdev N, Jalil RT, McMenemin R, Hughes S, Payne H, Green JS (2014) Second-line chemotherapy for advanced bladder cancer: a survey of current UK practice Urol Oncol, 32:52.e11-7.

Lamming DW, Ye L, Sabatini DM, Baur JA (2013) Rapalogs and mTOR inhibitors as anti-aging therapeutics. J Clin Invest, 123:980-989.

Laplante M, Sabatini DM (2012) mTOR signaling in growth control and disease. Cell, 149:274-93.

Laplante M, Sabatini DM (2013) Regulation of mTORC1 and its impact on gene expression at a glance. J Cell Sci, 126:1713-1719.

Latini DM, Lerner SP, Wade SW, Lee DW, Quale DZ (2010) Bladder Cancer Detection, Treatment and Outcomes: Opportunities and Challenges. Urology, 75:334-339.

Letašiová S, Medve'ová A, Šovčíková A, Dušinská M, Volkovová K, Mosoiu C, Bartonová A (2012) Bladder cancer, a review of the environmental risk factors. Environ Health, 11 Suppl 1:S11.

Li F, An S, Hou L, Chen P, Lei C, Tan W (2014) Red and processed meat intake and risk of bladder cancer: a meta-analysis. Int J Clin Exp Med, 7:2100-2110.

Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet, 16:64-67.

Lin JC , Liao SK, Lee EH, Hung MS, Sayion Y, Chen HC, Kang CC, Huang LS, Cherng JM (2009) Molecular events associated with epithelial to mesenchymal transition of nasopharyngeal carcinoma cells in the absence of Epstein-Barr virus genome. J Biomed Sci, 24; 16:105.

Liu P, Cheng H, Roberts TM, Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov, 8:627-644.

Liu X, Zhang W, Geng D, He J, Zhao Y, Yu L (2014) Clinical significance of fibroblast growth factor receptor-3 mutations in bladder cancer: a systematic review and meta-analysis. Genet Mol Res, 13:1109-20.

Makhlin I, Zhang J Long CJ, Devarajan K, Zhou Y, Klein-Szanto AJ, Huang M, Chernoff J, Boorjian SA (2010) The mTOR pathway affects proliferation and chemosensitivity of urothelial carcinoma cells and is upregulated in a subset of human bladder cancers. BJU Int, 108 (2 Pt 2):E84-90.

Macarulla T, Capdevila J, Perez-Garcia J, Ramos FJ, Elez ME, Markman B, Ruiz-Echarri M, Tabernero J (2009) New approaches and targets in advanced colorectal cancer. Eur J Cancer, 45 Suppl 1:79-88.

Martelli AM, Evangelisti C, Chiarini F, McCubrey JA (2010) The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. Oncotarget, 1:89-103.

McFarlane C, Plummer E, Thomas M (2006). Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent

mechanism. J Cell Physiol, 209:501-514.

Milowsky MI, Iyer G, Regazzi AM, Al-Ahmadie H, Gerst SR, Ostrovnaya I, Gellert LL, Kaplan R, Garcia-Grossman IR, Pendse D, Balar AV, Flaherty AM, Trout A, Solit DB, Bajorin DF (2013) Phase II study of everolimus in metastatic urothelial cancer. BJU Int, 112:462-470.

Mitra AP, Cote RJ (2009) Molecular pathogenesis and diagnostics of bladder cancer. Annu Rev Pathol, 4:251-285.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, Boyd M (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst, 5; 83(11):757-66.

Moschetta M, Reale A, Marasco C, Vacca A, Carratù MR (2014) Therapeutic targeting of the mTOR-signalling pathway in cancer: benefits and limitations. Br J Pharmacol, 171:3801-3813.

Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grünwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, Lebwohl D, Ravaud A (2008) Efficacy of everolimus in advanced renal cell carcinoma: a double-bind, randomized, placebo-controlled phase III trial. Lancet, 372(9637):449-56.

Murta-Nascimento C, Schmitz-Dräger BJ, Zeegers MP, Steineck G, Kogevinas M, Real FX, Malats N (2007) Epidemiology of urinary bladder cancer: from tumor development to patient's death. World J Urol, 25:285-95.

Muthukkumar S, Ramesh TM, Bondada S (1995) Rapamycin, a potent immunosuppressive drug, causes programmed cell death in B lymphoma cells. Transplantation, 60(3): 264-270.

Netto GJ (2013) Molecular genetics and genomics progress in urothelial bladder cancer. Semin Diagn Pathol, 30:313-20.

Niegisch G, Retz M, Thalgott M, Balabanov S, Honecker F, Ohlmann CH, Stöckle M, Bögemann M, Vom Dorp F, Gschwend J, Hartmann A, Ohmann C, Albers P (2015) Second-Line Treatment of Advanced Urothelial Cancer with Paclitaxel and Everolimus in a German Phase II Trial (AUO Trial AB 35/09). Oncology, 89(2):70-8.

Ohno S, Ohno Y, Nakada H, Suzuki N, Soma G, Inoue M (2006) Expression of Tn and sialyl-Tn antigens in endometrial cancer: its relationship with tumor-produced cyclooxygenase-2, tumor-infiltrated lymphocytes and patient prognosis. Anticancer Res, 26, 4047e4053.

Oliveira PA, Colaco A, De la Cruz P LF, Lopes C (2006) Experimental bladder carcinogenesis-rodent models. Exp Oncol, 281:2-11.

Oliveira PA., Palmeira C, Colaço A, De La Cruz P LF, Lopes C (2006) DNA content analysis, expression of Ki-67 and p53 in rat urothelial lesions induced by N-butyl-N-(4-hydroxybutyl) nitrosamine and treated with mitomycin C and Bacillus Calmette-Gue'rin. Anticancer Res, 26:2995–3004.

Oliveira PA, Arantes-Rodrigues R, Sousa-Diniz C, Colaço A, Lourenço L, De La Cruz LF, Da Silva VM, Afonso J, Lopes C, Santos L (2009) The effects of sirolimus on urothelial lesions chemically induced in ICR mice by BBN. Anticancer Res, 29 (8):3221-6.

Ozaki H, Matsuzaki H, Ando H, Kaji H, Nakanishi H, Ikehara Y, Narimatsu H (2012) Enhancement of metastatic ability by ectopic expression of ST6GalNAcI on a gastric cancer cell line in a mouse model. Clin. Exp. Metastasis, 29, 229e238.

Padrão AI, Oliveira P, Vitorino R, Colaço B, Pires MJ, Márquez M, Castellanos E, Neuparth MJ, Teixeira C, Costa C, Moreira-Gonçalves D, Cabral S, Duarte JA, Santos LL, Amado F, Ferreira R (2013) Bladder cancer-induced skeletal muscle wasting: disclosing the role of mitochondria plasticity. Int J Biochem Cell Biol, 45:1399-1409.

Palmeira C, Oliveira PA, Arantes-Rodrigues R, Colaço A, De la Cruz PL, Lopes C, Santos L (2009) DNA cytometry and kinetics of rat urothelial lesions during chemical carcinogenesis. Oncol Rep, 21 (1):247-52.

Palmeira C, Oliveira PA, Lameiras C, Amaro T, Silva VM, Lopes C, Santos L (2010) Biological similarities between murine chemical-induced and natural human bladder carcinogenesis. Oncol Lett, 1:373–7.

Pang H, Faber LE (2001) Estrogen and rapamycin effects on cell cycle progression in T47D breast cancer cells. Breast Cancer Res Treat, 70: 21-26.

Park SJ, Lee TJ, Chang IH (2011) Role of the mTOR Pathway in the Progression and Recurrence of Bladder Cancer: An Immunohistochemical Tissue Microarray Study. Korean J Urol, 52 (7):466-73.

Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, Gray NS, Sabatini DM (2009) DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell, 137:873-86.

Pinho S, Marcos NT, Ferreira B, Carvalho AS, Oliveira MJ, Santos-Silva F, Harduin-Lepers A, Reis CA (2007) Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett, 249, 157e 170.

Ploeg M, Aben KK, Kiemeney LA (2009) The present and future burden of urinary bladder cancer in the world. World J Urol, 27:289-93.

Pópulo H, Lopes JM, Soares P (2012) The mTOR signalling pathway in human cancer. Int J Mol Sci, 13:1886-918.

Porta C, Paglino C, Mosca A (2014) Targeting PI3K/Akt/motor Signaling in Cancer. Front Oncol, 14; 4:64.

Reis CA, OsorioH, Silva L, Gomes C, David L (2010) Alterations in glycosylation as biomarkers for cancer detection. J. Clin. Pathol, 63:322-329.

RON (2006) Registo Oncológico Nacional. [Internet]. Available from http://issuu.com/ipoporto/docs/ron_2006_print_reduce?e=7796583/2503360>. [Accessed July 2014].

RON (2008) Registo Oncológico Nacional. [Internet]. Available from http://issuu.com/ipoporto/docs/ron_2008_print_reduce?e=7796583/2503360>. [Accessed July 2014].

Rosfjord E, Lucas J, Li G, Gerber HP (2014) Advances in patient-derived tumor xenografts: from target identification to predicting clinical response rates in oncology. Biochem Pharmacol, 15: 91 (2): 135-43.

Rubio-Viqueira B, Hidalgo M (2009) Direct in vivo xenograft tumor model for predicting chemotherapeutic drug response in cancer patients. Clin Pharmacol Ther, 85:217-221.

Rushton L, Bagga S, Bevan R, Brown TP, Cherrie JW, Homes P, Fortunato L, Slack R, Van Tongeren M, Young C, Hutchings SJ (2010) Occupation and cancer in Britain. Br J Cancer, 102:1428-37.

Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She QB, Maurer M, Koujak S, Ferrando AA, Malmström P, Memeo L, Isola J, Bendahl PO,Rosen N, Hibshoosh H, Ringnér M, Borg A, Parsons R (2007) Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci U S A. 104(18):7564-9.

Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell, 78: 35-43.

Sabers CJ, Martin MM, Brunn GJ, Williams JM, Dumont FJ, Wiederrecht G, Abraham RT (1995) Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J Biol Chem, 270: 815-22.

Santos J, Chaves J, Videira M, Botelho M, Costa J, Oliveira J, Santos L (2012) *Schistosomiasis haematobium* and bladder cancer: Retrospective analysis of 145 patients admitted to the Urology. Department at the Américo Boavida Hospital, Luanda. Acta Urológica, 1: 15-20.

Sansal I, Sellers WR (2004) The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol, 2004 Jul 15; 22(14):2954-2963.

Sarbassov DD , Ali SM, Sabatini DM (2005) Growing roles for the mTOR pathway. Curr Opin Cell Biol, 17(6):596-603.

Sehgal SN (1998) Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem, 31: 335–340.

Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell, 22:159-168.

Sengupta S, Peterson TR, Sabatini DM (2010) Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell, 40:310–322.

Seront E, Rottey S, Sautois B, Kerger J, D'Hondt LA, Verschaeve V, Canon JL, Dopchie C, Vandenbulcke JM, Whenham N, Goeminne JC, Clausse M, Verhoeven D, Glorieux P, Branders S, Dupont P, Schoonjans J, Feron O, Machiels JP (2012) Phase II study of everolimus in patients with locally advanced or metastatic transitional cell carcinoma of the urothelial tract: clinical activity, molecular response, and biomarkers. Ann Oncol, 23:2663-2670.

Seufferlein,T, Rozengurt E (1996) Rapamycin inhibits constitutive p70s6k phosphorylation, cell proliferation, and colony formation in small cell lung cancer cells. Cancer Res, 56:3895-3897.

Shariat SF, Sfakianos JP, Droller MJ, Karakiewicz PI, Meryn S, Bochner BH (2010) The effect of age and gender on bladder cancer: a critical review of the literature. BJU Int, 105:300-308.

Sharma S, Ksheersagar P, Sharma P (2009) Diagnosis and Treatment of Bladder Cancer. Am Fam Physician, 80:717-723.

Sievert KD, Amend B, Nagele U, Schilling D, Bedke J, Horstmann M, Hennenlotter J, Kruck S, Stenzl A (2009) Economic aspects of bladder cancer: what are the benefits and costs? World J Urol, 27:295–300.

Sun CH, Chang YH, Pan CC (2011) Activation of the PI3K/Akt/mTOR pathway correlates with tumour progression and reduced survival in patients with urothelial carcinoma of the urinary bladder. Histopathology, 58:1054-63.

Talmadge JE (2007) Clonal selection of metastasis within the life history of a tumor. Cancer Res, 15; 67 (24):11471-5.

Tanaka T, Miyazawa K, Tsukamoto T, Kuno T, Suzuki K (2011) Pathobiology and chemoprevention of bladder cancer. J Oncol, 2011:528353.

Teply BA and Kim JJ (2014) Systemis therapy for bladder cancer – a medical oncologists perspective. J Solid Tumors , 4(2):25-35.

Tisdale MJ (2009) Mechanisms of cancer cachexia. Physiol Rev, 89:381-410.

Tsuchiya A, Kikuchi Y, Ando Y, Abe R (1995) Correlation between expression of sialosyl-T antigen and survival in patients with gastric cancer. Br J Surg, 82(7):960-2.

Turo R, Harnden P, Thygesen H, Fleischmann A, Thalmann GN, Seiler R, Cross WR, Knowles MAJ Urol (2015) FGFR3 Expression in Primary Invasive Bladder Cancers and Matched Lymph Node Metastases, 193:325-330.

Urquidi V, Rosser CJ, Goodison S (2012) Molecular diagnostic trends in urological cancer: biomarkers for non-invasive diagnosis. Curr Med Chem, 19:3653-3663.

van der Poel HG, Hanrahan C, Zhong H, Simons JW (2003) Rapamycin induces Smad activity in prostate cancer cell lines. Urol Res, 30: 380-386.

Vicier C, Dieci MV, Arnedos M, Delaloge S, Viens P, Andre F (2014) Clinical development of mTOR inhibitors in breast cancer. Breast Cancer Res, 16(1):203.

Vignot S, Faivre S, Aguirre D, Raymond E (2005) mTOR-targeted therapy of cancer with rapamycin derivatives. Ann Oncol, 16:525-37.

Vishnu P, Mathew J, Tan WW (2011) Current therapeutic strategies for invasive and metastatic bladder cancer. Onco Targets Ther, 4:97-113.

Wadhwa N, Jatawa SK, Tiwari A (2012) Non-invasive urine based tests for the detection of bladder cancer. J Clin Pathol, 65:970-975.

Weistenhofer W, Blaszkewicz M, Bolt HM, Golka K (2008) N-acetyltransferase-2 and medical history in bladder cancer cases with a suspected occupational disease (BK 1301) in Germany. J Toxicol Environ Health, 71:906-10.

Werther JL, Tatematsu M, Klein R, Kurihara M, Kumagai K, Llorens P, Guidugli Neto J, Bodian C, Pertsemlidis D, Yamachika T, Kitou T, Itzkowitz S (1996) Sialosyl-Tn antigen as a marker of gastric cancer progression: an international study. Int J Cancer, 21; 69(3):193-9.

Wilding JL, Bodmer WF (2014) Cancer cell lines for drug discovery and development. Cancer Res, 1; 74(9):2377-84.

Williams CR (2014) The combination of an mTORc1/TORc2 inhibitor with lapatinib is synergistic in bladder cancer in vitro. Urol Oncol, 32:317-26.

Willems L, Tamburini J, Chapuis N, Lacombe C, Mayeux P, Bouscary D (2012) PI3K and mTOR signaling pathways in cancer: new data on targeted therapies. Curr Oncol, 14(2):129-38.

Williams SG, Stein JP (2004) Molecular pathways in bladder cancer. Urol Res, 32: 373-385.

Wu X, Ros MM, Gu J, Kiemeney L (2008) Epidemiology and genetic susceptibility to bladder cancer. BJU Int, 102(9 Pt B):1207-1215.

Wu XR m (2005) Urothelial tumorigenesis: a tale of divergent pathways. Nat Rev Cancer, 5:713-725.

Wu X, Obata T, Khan Q, Highshaw RA, De Vere White R, Sweeney C (2004) The phosphatidylinositol-3 kinase pathway regulates bladder cancer cell invasion. BJU Int, 93(1):143-50.

Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in groth and mnetabolism. Cell 124(3):471-84.

Yang Q, Guan KL (2007) Expanding mTOR signaling. Cell Res, 17:666-81.

Ye F, Wang L, Castillo-Martin M, McBride R, Galsky MD, Zhu J, Boffetta P, Zhang DY, Cordon-Cardo C (2014) Biomarkers for bladder cancer management: present and future. Am J Clin Exp Urol, 2:1-14.

Youssef RF, Mitra AP, Bartsch G Jr, Jones PA, Skinner DG, Cote RJ (2009) Molecular targets and targeted therapies in bladder cancer management. World J Urol, 27:9-20.

Zarogoulidis P, Lampaki S, Turner JF, Huang H, Kakolyris S, Syrigos K, Zarogoulidis K (2014) mTOR pathway: A current, up-to-date mini-review (Review). Oncol Lett, 8(6):2367-2370.

Zask A, Verheijen JC, Richard DJ (2011) Recent advances in the discovery of small-molecule ATP competitive mTOR inhibitors: a patent review. Expert Opin Ther Pat, 21:1109-27.

Zaytseva YY, Valentino JD, Gulhati P, Evers BM (2012) mTOR inhibitors in cancer therapy. Cancer Lett, 319:1-7.

Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol, 12, 21–35.