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**ESTUDO DE BIOMARCADORES E
DO POLIMORFISMO DA M_nSOD EM
PACIENTES COM CÂNCER DE
BEXIGA.**

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BEXIGA.**

Tese submetida ao Programa de Pós-Graduação em Patologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

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LISTA DE ABREVIATURAS

Ala: alanina

BCG: Bacillus Calmette-Guerin

Ca de Bexiga: câncer de bexiga

CAT: catalase

CDK: proteínas cinases dependentes de ciclina

DCV: doença cardiovascular

DM Tipo 2: Diabetes Mellitus tipo 2

EC: extracelular

EN2: engrailed 2

EO: estresse oxidativo

EROs: espécies reativas de oxigênio

FGFR3: receptor do fator de crescimento de fibroblasto

GLOBOCAN: Agência Internacional de Pesquisa sobre Câncer

GPx: glutathione peroxidase

HGPUC: carcinoma urotelial papilar de alto grau

HPV: papilomavírus humano

H₂O₂: peróxido de hidrogênio

IHQ: imunoistoquímica

INCA: Instituto Nacional do Câncer

LGPUC: carcinoma urotelial papilar de baixo grau

LOO[•]: radical peroxila

LO[•]: radical alcóxila

MMP-1: metaloproteinase de matriz

MnSOD: superóxido dismutase dependente de manganês

MICB: câncer de bexiga que invade o músculo liso

Mx: metástases não avaliadas

M0: sem metástase distante

M1: com metástase distante

NF- κ : Fator nuclear κ B

NMICB: câncer de bexiga não-músculo-invasivo

NO $^{\circ}$: óxido nítrico

NOX: NAHPH oxidase

Nx: nodos não avaliados

N0: sem metástase

N1: metástase em um único nodo da pelve verdadeira

N2: metástase em múltiplos nodos da pelve verdadeira

N3: metástase em nodos linfáticos da íliaca comum

OH $^{\circ}$: hidroxila

OMS: Organização Mundial de Saúde

O $_2^{\circ}$: superóxido

PHS: *Physicians' Health Study*

PRx: peroxiredoxinas

pT: estágio patológico

PUNLMP: neoplasia urotelial papilar de baixo potencial maligno

RL: radicais livres

RTU: ressecção transuretral

SNP: polimorfismo de nucleotídeo simples ou polimorfismo de um único nucleotídeo

SODs: superóxido dismutases

SOD1: cobre/zinco SOD citosólica

SOD2: MnSOD mitocondrial

SOD3: CuZnSOD extracelular

Ta: carcinoma papilar não invasivo

TNM: TNM Classification of Malignant Tumours

Tis: carcinoma *in situ*

Tx: tumor não avaliado

T0: sem evidência de tumor

T1: invasão no tecido subepitelial

T2: invasão muscular

T3: invasão no tecido perivascular

T4: invasão a órgãos adjacentes

Val: Valina

VEGF: fator de crescimento endotelial vascular

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RESUMO

Introdução: O câncer de bexiga (Ca de bexiga) é a sétima (7ª) neoplasia mais comum em homens diagnosticados no mundo, caindo para décima primeira (11ª) quando ambos os sexos são observados. A suscetibilidade ao Ca de bexiga depende da associação de fatores de risco genéticos e ambientais, tais como exposição ocupacional, fator dietético e tabagismo. Espécies reativas de oxigênio (ERO), também presente na fumaça de cigarro, são constantemente geradas pelo organismo aeróbio como resultado do metabolismo normal. No entanto, um aumento do nível de ERO causa estresse oxidativo e cria um ambiente potencialmente tóxico para as células. As variações dos genes envolvidos no metabolismo das ERO são considerados potenciais fatores de risco no desenvolvimento do câncer, portanto, o presente estudo observou a associação entre o risco para o Ca de bexiga e as variantes genéticas da enzima antioxidante MnSOD. Além da associação do estresse oxidativo e a carcinogênese, nós revisamos e investigamos o uso de diferentes biomarcadores imunoistoquímicos (IHQ) no diagnóstico e prognóstico do Ca de bexiga, a fim de melhorar a escolha do tratamento, reduzir o número de cistoscopias e detectar a recorrência e progressão mais cedo que os testes tradicionais.

Métodos: Uma revisão, um estudo de coorte e um estudo de caso-controle foram conduzidos para avaliar a associação dos biomarcadores IHQ e o polimorfismo da MnSOD com o Ca de bexiga. Um total de 357 indivíduos foram incluídos, dos quais, 110 casos e 247 controles. De acordo com os bancos de dados do Pubmed, Medline e Cochrane Library, uma revisão foi realizada em “estudos experimentais sobre biomarcadores histopatológicos para câncer de bexiga não músculo invasivo” publicados no período entre 2009 e 2014. Para o estudo de coorte, 93 pacientes forneceram o tecido histopatológico para análises IHQ das seguintes proteínas: p16, p21, p27, p53, pRb e Ki67. No estudo de caso-controle todos os 357 indivíduos tiveram 5 ml de sangue coletado e fragmentos de DNA genômico contendo o polimorfismo MnSOD Ala9Val na sequência do gene humano SOD2, foram amplificados por PCR.

Resultados: Após revisão da literatura foi possível identificar os biomarcadores e dividi-los de acordo com a sua via de sinalização, fornecendo os achados obtidos, referência e amostra do estudo. Foi possível então identificar os marcadores considerados promissores (p53, Ki-67, CK20 e EN2) e avaliar seus potenciais valores prognóstico e preditivo em estudos com Ca de bexiga. No estudo de coorte, as principais categorias de estadiamento observadas nos pacientes foram T1 (53%) e Ta (29%) e a distribuição entre o grau tumoral foram de 58% baixo grau e 42% alto grau. As expressões IHQ de p16, p21, p27, p53, pRb e Ki-67 foram alteradas em 31%, 42%, 60%, 91%, 27% e 56%, respectivamente. A expressão IHQ de Ki-67 foi associada com grau histológico ($p = 0,016$) e expressão de pRb com a sobrevida livre de recidiva ($p = 0,035$). Nenhum marcador foi estatisticamente significativo na análise multivariada, porém o número de marcadores alterados mostrou-se relacionado à sobrevida livre de recidiva ($p = 0,004$).

No estudo de caso-controle nós encontramos um importante aumento do risco no desenvolvimento do câncer de bexiga entre os indivíduos com o alelo Ala comparados aos portadores do alelo Val (OR = 2,31). O sexo masculino, como já reportado por outros autores, é um fator de risco associado ao Ca de bexiga e os principais estadiamentos observados foram o Ta (24,5%) e T1 (50,9%). Nós também observamos uma tendência da correlação do genótipo Val/Val e a ocorrência de Ca de bexiga não músculo invasivo.

Conclusão: O Ca de bexiga é uma neoplasia heterogênea que apresenta alta probabilidade de recorrência e progressão. Os biomarcadores atuam neste contexto para aumentar a precisão do prognóstico e consequentemente permitir uma modulação adequada da doença. No entanto, as anormalidades moleculares do Ca de bexiga são altamente complexas, sendo assim pouco provável que um único marcador seja capaz de identificar e estratificar pacientes em categorias prognósticas precisas. Como demonstrado pelos nossos dois estudos, de revisão e coorte, a combinação de marcadores independentes, porém complementares, pode permitir um diagnóstico ou prognóstico mais preciso que um marcador isolado, sendo um tópico importante para investigação a pesquisa por um painel de biomarcadores IHQ para o Ca de bexiga. Em adição, sabe-se que ERO podem desempenhar um papel importante na carcinogênese

da bexiga, e a susceptibilidade individual ao câncer pode ser modulada pelo polimorfismo da MnSOD. Este estudo demonstrou que a genótipo Ala/Ala pode ser considerada como um fator de risco para o câncer da bexiga e que novos estudos devem ser feitos correlacionando estresse oxidativo, fatores ambientais e genéticos ao risco de recorrência, progressão e desenvolvimento do Ca de bexiga.

Palavras chaves: Câncer de Bexiga, Estresse oxidativo, Polimorfismo, MnSOD, Biomarcadores.

1 INTRODUÇÃO

1.1 Câncer de Bexiga e Aspectos Epidemiológicos

O câncer de bexiga (Ca de bexiga), das neoplasias do homem, é a sétima (7ª) mais comumente diagnosticada no mundo, caindo para décima primeira (11ª) quando ambos os sexos são observados, tendo cerca de 429.793 novos casos diagnosticados em 2012. As maiores taxas de incidência são observadas nos países desenvolvidos, que respondem por 59% dos diagnósticos anuais. Já a mortalidade mundial verificada em 165.068 óbitos em 2012, é levemente superior nos países menos desenvolvidos (Figura 1). Para o continente americano, estima-se ter havido 101.593 novos casos e 28.739 óbitos em 2012, sendo o quinto tipo de câncer mais incidente no local. Dados obtidos sobre incidência e mortalidade no continente americano através da Agência Internacional de Pesquisa sobre Câncer (GLOBOCAN) são apresentados na figura 2 (Ferlay e cols., 2012).

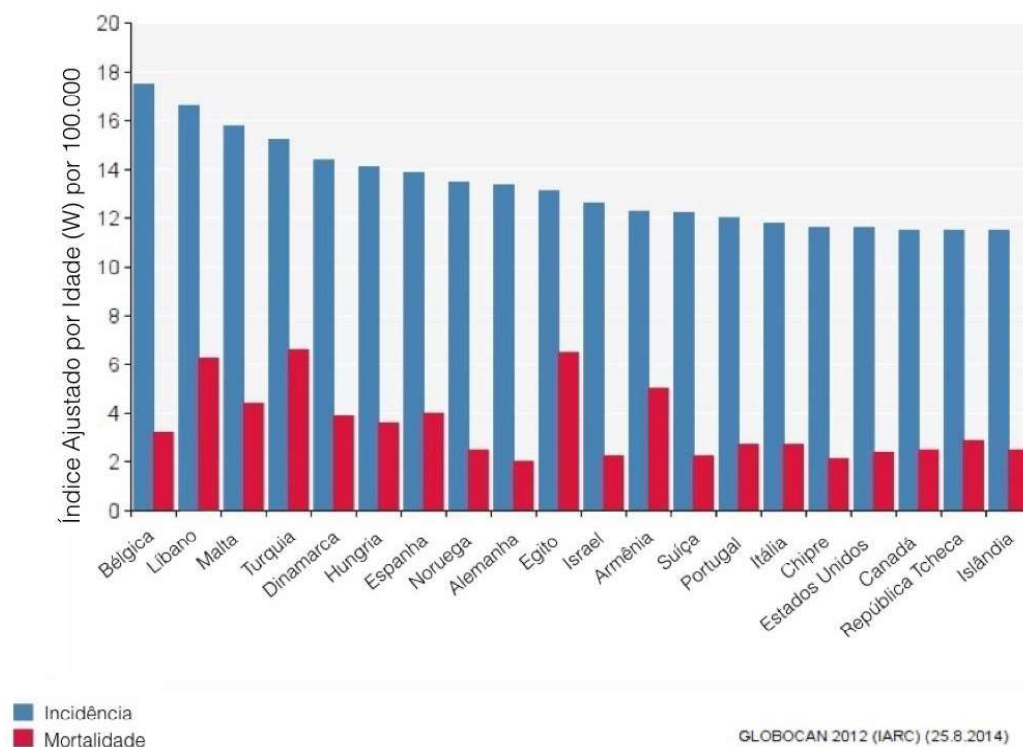


Figura 1: Estimativas de taxas de incidência e mortalidade padronizadas por idade (20 maiores taxas no mundo) por 100.000 habitantes em ambos os sexos para câncer de bexiga (adaptada de GLOBOCAN).

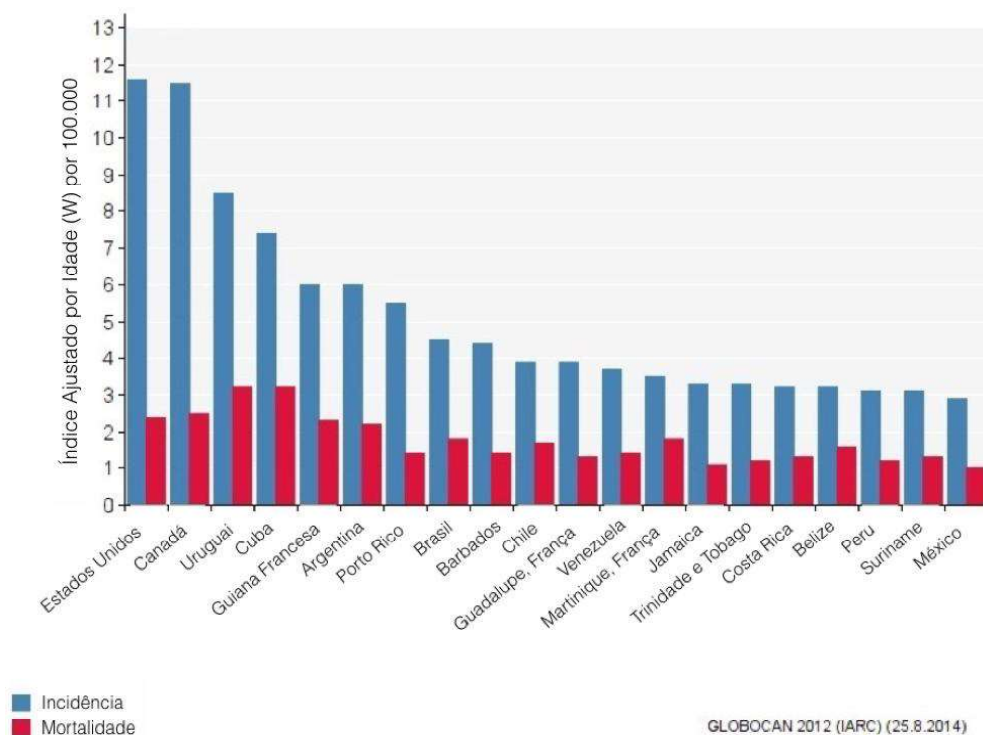


Figura 2: Estimativas de taxas de incidências e mortalidades americanas padronizada por idade por 100.000 habitantes em ambos os sexos para câncer de bexiga (adaptada de GLOBOCAN).

No Brasil, segundo dados GLOBOCAN 2012, o Ca de bexiga apresentou uma incidência de 9.924 casos novos, sendo 7.047 casos novos no gênero masculino e 2.877 casos feminino. A mortalidade na população brasileira foi estimada em 4.021 óbitos, sendo 2.805 entre os homens e 1.216 entre as mulheres. De acordo com o Instituto Nacional do Câncer (INCA) a estimativa de novos casos para 2016 na população são 9.670 (Ferlay e cols., 2012; INCA, 2016).

Dados da União Européia relatam que a taxa de incidência padronizada por sexo é de 27 a cada 100.000 homens e 6 a cada 100.000 mulheres. A proporção de casos de neoplasia vesical entre homens e mulheres é de 3:1. Apesar da maior prevalência no sexo masculino, a mortalidade é cerca de 40% menor neste grupo, pois a doença tem 32% mais chances de ser diagnosticada em estágios iniciais, em relação aos casos no sexo feminino. Isto se deve ao retardo do diagnóstico pelo equívoco dos sintomas iniciais como hematúria e

irritação vesical com infecção do trato urinário comum entre as mulheres. Outro fator que pode contribuir para este fenômeno é o anatômico, pois nos homens a presença da próstata e do músculo detrusor espessado dificulta a disseminação da neoplasia (Kumar e cols., 2010; Babjuk e cols., 2013).

Etnia e idade são outras variáveis importantes na epidemiologia do Ca de bexiga. Americanos caucasianos têm aproximadamente o dobro de risco de desenvolver câncer de bexiga quando comparados a afro-americanos. Latinos apresentam um risco ainda menor de desenvolver câncer de bexiga quando comparados a afro-americanos (American Cancer Society, 2006).

O Ca de bexiga é uma doença predominantemente encontrada em idosos, com mais de 90% dos diagnósticos em pacientes acima de 55 anos; mesmo incomum, o Ca de bexiga pode ocorrer em adultos jovens e até mesmo em crianças. A média etária do diagnóstico inicial é de 65 anos (American Cancer Society, 2006; Kumar e cols. 2010).

A incidência do câncer de bexiga apresenta-se menor em alguns registros mundiais, isso tem sido interpretado devido a diminuição da exposição aos agentes causadores - principalmente a diminuição do fumo e melhor higiene ocupacional. Não existe uma tendência uniforme, porém, as taxas de incidência padronizada no Reino Unido diminuíram, enquanto as taxas de incidência nos americanos brancos permanecem estáveis no mesmo período de tempo (Bosetti e cols., 2011; Burger e cols., 2013a).

1.2 Fatores de risco

Alguns fatores de risco que predispõem o Ca de bexiga já se encontram bem definidos como o risco ocupacional, tabagismo e a suscetibilidade genética. O risco ocupacional é visto como o segundo fator mais importante no desenvolvimento de Ca de bexiga urotelial, ocorrendo principalmente quando o indivíduo se expõe a aminas aromáticas, hidrocarbonetos policíclicos aromáticos e hidrocarbonetos clorados – todos utilizados em áreas industriais de processamento de tintas, tecidos, produtos de metais e petróleo (Burger e cols., 2013a).

A exposição à radiação ionizante está relacionada ao risco aumentado para Ca de bexiga. A radiação ionizante tem uso industrial, militar e na área médica, como por exemplo, a utilização de tomografia computadorizada, medicina nuclear e na radioterapia para o tratamento de cânceres de próstata, reto e colo uterino. (Babjuk e cols., 2013).

O tabagismo é o principal fator de risco estabelecido em ambos os sexos, responsável por 50% dos tumores masculinos e 35% dos femininos. De fato, os fumantes têm de 2 a 4 vezes maior risco de desenvolver Ca de bexiga do que os não fumantes e o risco aumenta quando a intensidade e/ou a duração do fumo são aumentadas (Zeegers e cols., 2000; Kirkali e cols., 2005; Silvermann e cols., 2006).

Das substâncias encontradas no cigarro, as nitrosaminas e as alfa e beta-naftilaminas, que são secretadas na urina de fumantes, apresentam efeito carcinogênico conhecido sobre o urotélio. Já o 4-aminobifenil e o benzopireno são metabolizados por enzimas hepáticas, produzindo compostos com alta afinidade pela molécula de DNA e que, portanto, tornam-se agentes mutagênicos em potencial (Madeb e cols., 2004; Bosseti e cols., 2011).

O uso do tabaco e a exposição ocupacional são os maiores fatores de risco nos países desenvolvidos, enquanto que a infecção crônica pelo *Schistosoma haematobium* em países da África (particularmente o Egito) e do Oriente Médio são responsáveis por cerca de 50% dos casos. A maioria dos cânceres de bexiga associados ao *S. haematobium* são carcinomas epidermóides, enquanto que aqueles associados ao tabaco são carcinomas uroteliais (Parkin e cols., 2006; Burger e cols., 2013a).

Dados recentes do Atlas do Genoma Humano, indicaram que o Ca de bexiga abriga um grande número de mutações genômicas e que, muitas delas, não estão relacionadas ao tabagismo. Este estudo revelou mutações em 32 genes, incluindo múltiplos genes envolvidos na regulação do ciclo celular, regulação da cromatina e na via de sinalização das quinases, bem como outros 9 genes não previamente relatados como potenciais mutagênicos em qualquer tipo de câncer. O sequenciamento completo de genoma e de RNA identificaram

ativação recorrente enquadrando fusões e expressões ou integrações do FGFR3-TACC3 com diferentes vírus (incluindo o HPV subtipo 16) que estão associados à inativação gênica (The Cancer Genome Atlas Research Network, 2014).

1.3 Carcinoma urotelial de bexiga

As neoplasias malignas de bexiga são derivadas do epitélio transicional ou urotelial em cerca de 95% dos casos, sendo o mesmo tecido que reveste as vias urinárias da pelve renal à uretra proximal. Os outros 5% correspondem aos tumores mesenquimais, o mais comum é o leiomioma, originário do tecido muscular (Kumar e cols., 2010; Babjuk e cols., 2013; Tavora e cols., 2013).

O carcinoma urotelial de bexiga pode apresentar-se como um tumor não invasivo papilar, projetando-se da superfície da mucosa para a luz vesical, sendo facilmente ressecável. Embora estes tumores superficiais frequentemente recidivem, eles geralmente não progridem com invasão da parede vesical nem geram metástase. No entanto, cerca de um terço dos cânceres de bexiga apresentam-se como tumores sólidos, não papilares, originados de carcinoma *in situ*. Esses tumores invadem a parede da bexiga e têm uma alta propensão a metástase. Para estes tumores invasivos, 5 anos de sobrevida, em pacientes submetidos à cistectomia radical, é observada em 58% dos casos. Essa diferença na morfologia e sobrevida implica distintos caminhos para o câncer não invasivo vs câncer que invade o músculo liso (Dinney e cols., 2004; Speiss e Czerniak, 2006; Malkowicz e cols., 2007; Nuhn e cols., 2012; Tavora e cols., 2013).

Aproximadamente 75% dos pacientes com Ca de bexiga apresentam uma doença que está confinada à mucosa (estádio Ta , Tis) ou submucosa (estádio T1). Essas categorias são agrupadas como tumores de bexiga não-músculo-invasivo (NMICB). O NMICB tem uma alta prevalência devido às baixas taxas de progressão e uma sobrevivência de longo prazo em muitos casos. Pacientes com Ca de bexiga músculo que invadem o músculo liso (MICB) estão em maior risco de mortalidade específica por câncer. Os NMICB, tendem a recidivar com

relativa frequência (60-70%), progredindo para MICB em 20 a 30% dos casos (Parekh e cols., 2006; Babjuk e cols., 2013).

Pacientes com doença em estágio T1 (com invasão da lâmina própria), de alto grau e com presença de carcinoma *in situ* associado, apresentam uma maior chance de evoluir para doença invasiva do músculo liso ou metastática durante o seguimento da doença (Stein e Penson, 2008).

Os tumores de baixo grau e do tipo papilíferos (não-músculo-invasivos) apresentam alterações no cromossomo 9, na proteína reguladora do ciclo celular, ciclina D1 e no receptor do fator de crescimento de fibroblasto, FGFR3. Apresentam ainda uma produção excessiva do fator de crescimento endotelial vascular (VEGF), promotor da angiogênese. Por outro lado, alterações nas proteínas reguladoras p53 e pRb são comumente identificadas no carcinoma *in situ* e em formas invasivas do Ca de bexiga (Gontero e cols., 2001).

A detecção precoce é a melhor forma para se obter uma maior probabilidade de cura, evitar o desenvolvimento de metástases e prolongar o tempo de vida. Desta maneira a maioria dos cânceres de bexiga identificada precocemente apresenta um bom prognóstico, ao contrário daqueles detectados em uma fase mais avançada da doença na qual o tumor já adquiriu capacidade de invasão (Theodorescu, 2003).

1.4 Grau e Estadiamento

O Ca de bexiga possui duas versões usuais de classificação, segundo a Organização Mundial de Saúde (OMS): uma publicada em 1973, que o classifica em papiloma urotelial e carcinoma urotelial de graus 1, 2 ou 3; e outra publicada em 2004, que classifica o Ca de bexiga em papiloma, neoplasia urotelial papilar de baixo potencial maligno (PUNLMP), carcinoma urotelial papilar de baixo grau (LGPUC) ou carcinoma urotelial papilar de alto grau (HGPUC). A versão mais recente de classificação reconhece a existência de diferentes tipos de tumores não-invasivos, o que permite uma estratificação em categorias de prognóstico mais significativas. Entretanto, considera-se ambas versões (Figura 3b)

equivalentes em reprodutibilidade e valor prognóstico (Miyamoto e cols., 2010; May e cols., 2010; Babjuk e cols., 2013).

Há ainda o estadiamento TNM, outro tipo de classificação que avalia o tamanho e a extensão da neoplasia a partir do tumor primário, dos linfonodos regionais e da presença de metástase, o que auxilia na identificação do tratamento mais adequado. O tumor primário, representado pela letra T, pode ser classificado progressivamente em Tx (tumor não avaliado), T0 (sem evidências), Ta (carcinoma papilar não invasivo), Tis (carcinoma *in situ*), T1 (invasão no tecido subepitelial), T2 (invasão muscular), T3 (invasão no tecido perivesical) e T4 (invasão a órgãos adjacentes). O estadiamento T está ilustrado na Figura 3a. Os linfonodos regionais, representados por N, são classificados em Nx (linfonodos não avaliados), N0 (sem metástase), N1 (metástase em um único linfonodo da pelve verdadeira), N2 (metástase em múltiplos linfonodos da pelve verdadeira), e em N3 (metástase em linfonodos da íliaca comum). A presença de metástase em outras partes do corpo, por sua vez, é representada por M e classificada em Mx (não avaliada), M0 (sem metástase distante) e M1 (com metástase distante) (Cheng e cols., 2009; Babjuk e cols, 2013; Hafeez e Huddart, 2013; Lamm e cols., 2014).

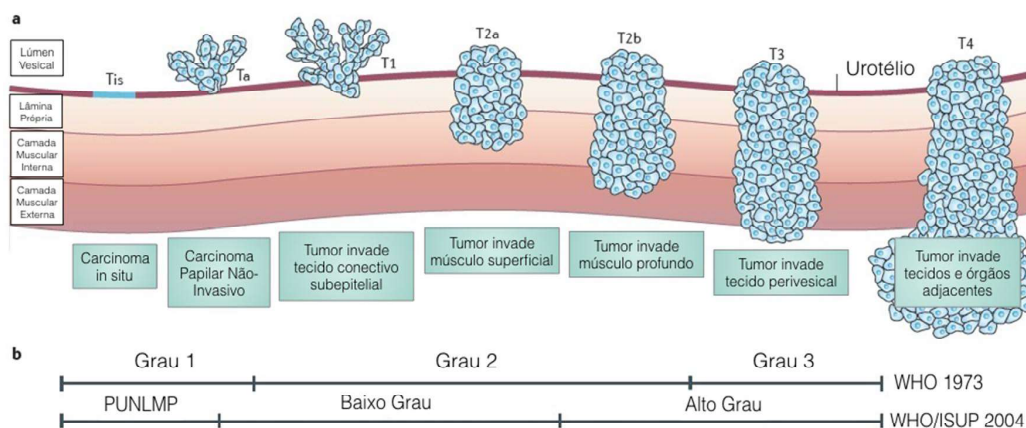


Figura 3: Câncer de bexiga grau e estadiamento (Adaptada de Knowles & Hurst 2015).

1.5 Diagnóstico

Os pacientes com a doença podem ser sintomáticos ou assintomáticos, os sintomas mais observados são: hematúria (achado mais comum), urgência,

disúria, aumento da frequência e dor pélvica. Dor pélvica e todos os sintomas relacionados à obstrução do trato urinário são vistos em tumores mais avançados. O Protocolo de Condutas para Hematúria Microscópica Assintomática da *American Urological Association* em 2001 recomenda que todos os pacientes com hematúria, principalmente aqueles sem evidência de infecção, cálculos ou outros fatores causais, devem se submeter a cistoscopia e realizar exames de imagem do trato urinário superior. Sintomas de irritação miccional incluindo aumento da frequência, urgência e disúria são associados principalmente ao carcinoma *in situ*. O diagnóstico do Ca de bexiga é considerado em pacientes com sintomas de irritação miccional na ausência de infecção (Smith e cols., 1999; Hall e cols., 2007; Grossfeld e cols., 2001; Witjes e cols., 2015).

O exame físico de pacientes com Ca de bexiga é frequentemente normal, sobretudo no caso de doença não-músculo-invasiva. Um exame bimanual no momento da ressecção transureteral (RTU) pode ajudar no estadiamento clínico, principalmente para pacientes com doença invasiva do músculo liso. Entretanto, considerando a discrepância entre o exame bimanual e o estadiamento após a cistectomia (11% de super estadiamento e 32% de baixo estadiamento), a interpretação do exame bimanual deverá ser cautelosa (Hall e cols., 2007; Ploeg e cols., 2012).

Imagem radiológica é realizada de maneira complementar à cistoscopia, fazendo parte da avaliação da hematúria em pacientes sob suspeita de câncer urotelial. Ainda, em pacientes com história pregressa de câncer de bexiga, a imagem pode ser útil na avaliação da presença de tumores do trato superior, que ocorrem em menos de 5% dos pacientes com história pregressa de câncer de trato inferior. A rotina de estudo de imagem inclui ultrassonografia, urografia intravenosa, tomografia computadorizada e ressonância magnética (Messing e Catalona, 1998; Hall e cols., 2007).

O diagnóstico do Ca de bexiga é feito pelo exame cistoscópico da bexiga e posterior avaliação histológica do tecido ressecado. Uma descrição cuidadosa dos achados cistoscópicos é necessária, devendo incluir a documentação do

local, tamanho, número e aparência (papilar ou sólido) dos tumores, bem como uma descrição de anomalias na mucosa (Witjes e cols., 2015).

O objetivo da RTU é permitir o diagnóstico histopatológico e estadiamento do tumor, o que requer a inclusão do músculo liso da bexiga nas ressecções. A estratégia da ressecção depende do tamanho da lesão, os tumores pequenos (menos de 1 cm) podem ser ressecados em bloco, contendo a amostra completa do tumor, mais uma parte subjacente da parede da bexiga incluindo o músculo. Tumores maiores têm de ser feita uma ressecção separada, em frações, incluindo a parte exofítica do tumor, a parede subjacente da bexiga com o músculo detrusor e as bordas da área de ressecção. Ao patologista deve ser encaminhada, pelo menos, a parte mais profunda da ressecção, permitindo-lhe fazer um diagnóstico correto (Burger e cols., 2013b).

1.5.1 Biomarcadores

Biomarcadores são substâncias que quando avaliadas podem indicar se um processo biológico é normal ou patológico, ou se há resposta farmacológica a determinada intervenção terapêutica (Ogata e cols., 2012).

Quanto a terminologia, um biomarcador prognóstico fornece informação sobre o desfecho global da patologia nos pacientes, independente de terapia. A presença ou ausência de determinado marcador de prognóstico pode ser útil na seleção de pacientes para certo tratamento, mas não pode prever a resposta individual ao tratamento de escolha. Marcadores prognósticos podem ser divididos em dois grupos: biomarcadores que fornecem informação sobre a recorrência do tumor em pacientes que recebem tratamento curativo e biomarcadores que se relacionam com a duração de sobrevida (sem progressão de doença) em pacientes com doença metastática. Um biomarcador com valor preditivo dá informação sobre o efeito de uma intervenção terapêutica em determinado paciente. Um biomarcador preditivo pode também ser alvo para a terapia (Oldenhuis e cols., 2008).

1.5.1.1 Biomarcadores Imunoistoquímicos

O padrão-ouro para diagnóstico de Ca de bexiga é a combinação de RTU ou biópsia do tumor e coloração histológica com eosina e hematoxilina junto a biomarcadores imunoistoquímicos (IHQ). A técnica de IHQ é amplamente utilizada em laboratórios de patologia ao redor do mundo. Estudos nessa área podem ser aplicados facilmente na clínica para manejo do paciente com câncer (Kim e cols., 2015; Ranzi e cols., 2015).

Biomarcadores IHQ são potencialmente úteis em relação às neoplasias quando na detecção de proteínas reguladoras do ciclo celular. Isso porque o controle do ciclo celular ocorre por meio de pontos de controle entre as etapas, os chamados *checkpoints*, que monitoram a disponibilidade de fatores de transcrição e a presença de lesões no DNA. A transição entre as fases é desencadeada pelo aumento na atividade das proteínas inibidoras de cinases dependentes de ciclina (CDK), como a p16, p21, p27 e a p53 responsáveis por fosforilar e, portanto, modular a atividade de diferentes subgrupos de proteínas-alvo específicas para a progressão do ciclo. Uma dessas proteínas-alvo é a pRb, que atua como um supressor do ciclo celular. As proteínas inibidoras de CDK são capazes de bloquear o ciclo celular em virtude de lesões no DNA. Assim, a ocorrência de qualquer mutação nos genes destas proteínas reguladoras, pode alterar as suas expressões e levar à ineficiência e desregulação do ciclo. Como consequência, tem-se uma proliferação celular descontrolada, principal característica do desenvolvimento neoplásico (Olsson *et al.*, 2012; Brandt *et al.*, 2009; Xylinas *et al.*, 2014; Matsushita *et al.*, 2011; Lotan *et al.*, 2013).

Em pacientes com história de NMICB, a sobrevida é uma área importante para o uso de novos marcadores. Isso se deve em grande parte as altas taxas de prevalência e recorrência da doença. Em tese, neste cenário de vigilância, um marcador pode tanto reduzir a quantidade de cistoscopias quanto detectar recorrência da progressão tumoral antes de testes tradicionais (Xylinas e cols., 2014).

Ranzi e colaboradores revisaram os biomarcadores imunoistoquímicos prognósticos utilizados atualmente e alguns biomarcadores promissores

relacionados ao NMICB. Os autores concluíram que a combinação de marcadores, incluindo p53, Ki-67, e CK20, com novos marcadores como o EN2 podem melhorar a estratificação do risco e proporcionar um prognóstico mais preciso das recorrências associadas ao Ca de bexiga (Ranzi e cols., 2015).

1.6 Tratamento

A escolha do tratamento para o Ca de bexiga é muito variável e decorre da sua probabilidade em recorrer ou progredir, de acordo com o grau de evolução que o tumor apresenta, assim o tratamento varia de um espectro mais simples onde se realiza somente a RTU (sem tratamento adjuvante), RTU e tratamento com BCG (*Bacillus Calmette-Guerin*) ou quimioterapia tópicas, até situações graves onde a ressecção completa da bexiga (cistectomia radical) é necessária. Para doença não-músculo invasiva, em que há alta probabilidade de recorrência, mas baixa de progressão, o tratamento padrão é a RTU completa, acrescida de quimioterapia intravesical adjuvante. Como a propensão à recorrência é alta, estes pacientes passam a ter um acompanhamento contínuo, com avaliação citológica e cistoscópica após o tratamento. Para doença invasiva do músculo liso, o tratamento padrão é a cistectomia radical, comumente acrescida de linfadenectomia pélvica. A reconstrução do trato urinário se faz com neobexiga intestinal ou conduto ileal, dependendo das condições clínicas do paciente. Em doença metastática e avançada, o tratamento quimioterápico associado ou não à cirurgia é o tratamento preconizado (Bellmunt e cols., 2011; Stenzl e cols., 2011; Ismaili e cols., 2011; Cody e cols., 2012; Babjuk e cols., 2013; Goodison e cols., 2013)

1.7 Espécies Reativas

Radicais livres (RL) são átomos ou moléculas que possuem um ou mais elétrons desemparelhados em seu orbital mais externo da eletrosfera, conferindo assim, um alto poder de reatividade. Outras moléculas além do oxigênio, como o carbono, enxofre e o nitrogênio também podem produzir RL ou espécies reativas, no entanto, o oxigênio é o que mais recebe atenção, dada sua importância nos processos metabólicos celulares (Halliwell, 2006; Dröse e Brandt, 2012; Shao e cols., 2012).

Radicais livres têm vida muito curta (em mili, micro ou nanosegundos) reagindo prontamente com lipídios, DNA e proteínas, causando danos e gerando produtos prejudiciais, como peróxidos de lipídios. O consequente dano às proteínas resulta em perda de atividade enzimática, enquanto o dano ao DNA pode resultar em mutagênese e carcinogênese (Dupont e Huecksteadt, 1992).

Espécies reativas de oxigênio (ERO) são constantemente geradas por organismo aeróbios por consequência do metabolismo normal. ERO incluem radicais livres de oxigênio [ex. superóxido ($O_2^{\cdot-}$), radical hidroxila ($OH^{\cdot}$), óxido nítrico ($NO^{\cdot}$), radicais alcóxila ($LO^{\cdot}$) e peróxila ($LOO^{\cdot}$)] assim como espécies não-radicais (ex. peróxido de hidrogênio, hidroperóxidos orgânicos e hipoclorido). ERO são indispensáveis para diversos processos fisiológicos da célula, incluindo proliferação celular, apoptose, ciclo celular e senescência celular. Entretanto, um nível aumentado de ERO causa estresse oxidativo e cria um ambiente potencialmente tóxico para as células (Storz, 2005).

Adicionalmente ao dano direto a moléculas e tecidos biológicos, o estresse oxidativo pode também ativar fatores de transcrição como o fator nuclear κB (NF- κB), cujo gatilho de cascata resulta na liberação de citocinas e promoção do processo inflamatório. Em condições fisiológicas normais, homeostase celular, um balanço entre geração de ERO e defesas oxidativas estão presentes na célula (Bag e Bag; 2008; García-Bailo e cols., 2011).

Radicais de oxigênio são poderosos agentes danosos ao DNA. ERO causam quebras de sequência, alterações nas bases de guanina e timina e permutas de cromátides irmãs, podendo inativar genes supressores tumorais dentro das células neoplásicas, ou ainda, aumentar a expressão de proto-oncogenes. Intermediários gerados pela oxidação de lipídios e proteínas podem formar alterações de DNA, que se não forem reparadas, podem conduzir a mutações. A extensão do dano induzido por ERO pode ser exacerbado pela diminuição da eficiência de mecanismos de defesa antioxidantes. Antioxidantes têm mostrado inibir a iniciação e promoção na carcinogênese, e neutralizam a imortalidade e transformação celular (Mates e cols., 1999; Marnett, 2000; Brown e Bicknell, 2001; Badjatia e cols., 2010).

O sistema antioxidante compreende um extenso grupo de enzimas endógenas e compostos exógenos e endógenos, que protegem contra o dano oxidativo impedindo a formação de espécies reativas, neutralizando e removendo-as, inibindo cadeias de reação oxidativa, quelando metais ou reparando danos em moléculas biológicas (Da Costa e cols., 2012).

A habilidade de administrar o estresse oxidativo é dependente do funcionamento de sistemas endógenos e exógenos de defesa antioxidante, sendo que ambos podem ser influenciados pela variação genética individual. Polimorfismo de nucleotídeo simples (SNPs) em genes que codificam enzimas antioxidantes endógenas ou a presença de antioxidantes na dieta, podem ter impacto direto no equilíbrio e prevenção do estresse oxidativo, favorecendo o desenvolvimento de câncer e doenças no indivíduo (Figura 4) (Da Costa e cols., 2012).

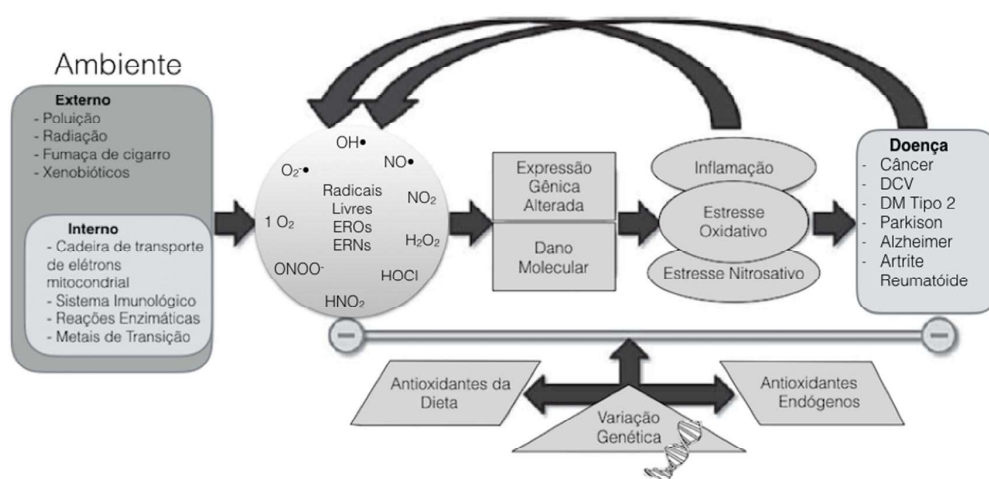


Figura 4: Resumo da relação entre a produção de EROs, estresse oxidativo, desenvolvimento de doenças e o papel de antioxidantes e da variação genética. Um acúmulo de EROs a partir de estímulo externo e interno pode causar dano molecular e resultar em estresse nitrosativo e oxidativo. EROs podem também alterar a expressão gênica, conduzindo à liberação de citocinas e inflamação, que resulta em mais produção EROs e espécies reativas de nitrogênio (ERNs). Inflamação e estresse oxidativo podem contribuir para o desenvolvimento de doenças crônicas e produção extra de espécies reativas. Antioxidantes endógenos e da dieta trabalham juntos para reduzir o desenvolvimento de estresse oxidativo e dano; sua função é ainda modificada pela variação genética individual. CVD = Doença cardiovascular; T2DM = Diabetes Mellitus Tipo 2. (Adaptada de Da Costa e cols., 2012)

1.8 Estresse oxidativo e câncer

Estresse oxidativo (EO), foi definido em 1991 como: um distúrbio no equilíbrio pró-oxidante - antioxidante em favor do primeiro, levando a um potencial dano oxidativo (Sies, 1991). Halliwell tem definido EO como: dano biomolecular causado pelo ataque de espécies reativas sobre compostos de organismos vivos (Halliwell e Whiteman, 2004).

ERO podem alcançar níveis excessivos como um resultado de desequilíbrio entre as duas forças opostas: produção de ERO e antioxidantes, particularmente sob situações patológicas e exposição a agentes tóxicos. ERO em excesso oxidam macromoléculas como o DNA, proteínas e lipídios, causando aumento da taxa de mutações, dano a organelas celulares e em situações extremas, morte celular. Essa situação de estresse é conhecida por ter papel fundamental no desenvolvimento de câncer (Imlay e cols, 1988; Imlay 2003; Bag e Bag, 2008; Maynard e cols., 2009).

O estresse oxidativo pode ainda conduzir a angiogênese do tumor. ERO aumentam a produção celular de fatores angiogênicos IL-8, VEGF e aumentam a secreção de metaloproteinase de matriz (MMP-1), a collagenase que auxilia no crescimento de vasos sanguíneos junto ao microambiente tumoral. Ampliando dessa forma, a migração de células tumorais e aumentando o risco de invasão e metástases. (Toyokuni e cols., 1995; Marnett, 2000; Kong e cols., 2000; Brown e Bicknell, 2001; Das, 2002; Valko e cols., 2004; Kumar e cols., 2008; Ushio-Fukai e Nakamura, 2008; Nishikawa, 2008).

Desta forma, o estresse oxidativo é associado à ocorrência de processos neoplásicos no organismo, sendo considerado atualmente um importante fator de risco para a carcinogênese. Fatores genéticos, ambientais ou a interação de ambos promovem uma ampla modulação na fisiologia celular e na carcinogênese (Spencer e cols., 2002; Valko e cols., 2007; Bakalova e cols., 2013).

1.9 Defesas Antioxidantes

Nas células eucarióticas, ERO são geradas por diversos processos metabólicos, incluindo a respiração mitocondrial e reações catalisadas por enzimas como: a NADPH oxidase (NOX), xantina oxidase e citocromo P450. A respiração mitocondrial é a maior fonte de ERO, como resultado da produção de $O_2^{\cdot-}$ pelos complexos I e III da cadeia de transporte de elétrons, cuja estimativa representa 1-2% do consumo de oxigênio pela célula. O radical superóxido é ainda convertido em outras ERO como $OH^{\cdot}$ e H_2O_2 (Dansen e Wirtz, 2001; Stowe e Camara, 2009).

A conversão de $O_2^{\cdot-}$ é catalisada pelas enzimas superóxidos dismutases (SODs), dando origem a H_2O_2 e oxigênio molecular. A H_2O_2 é ainda convertida em água e oxigênio em uma reação catabólica mediada pela catalase (CAT), peroxiredoxina (PRx) e glutathiona peroxidase (GPx), figura 5 (Che e cols., 2015).

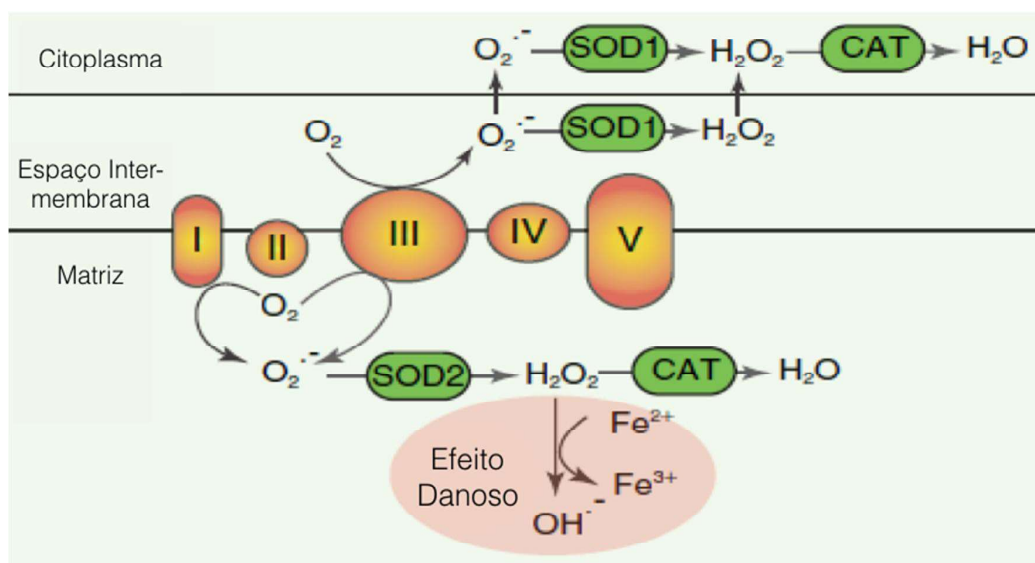


Figura 5: Sistemas Antioxidantes Celulares. SODs e CAT, as quais são independente de NADPH. A SOD1 é responsável por processar $O_2^{\cdot-}$ gerado no espaço intermembrana da mitocôndria pela cadeia transportadora de elétrons e de outras localidades do citoplasma. SOD2 é responsável por processar $O_2^{\cdot-}$ na matriz mitocondrial. A CAT ainda catalisa a reação de H_2O_2 para H_2O e O_2 (Adaptada de Che e cols., 2015).

Importante papel na defesa celular contra o estresse oxidativo é exercido por enzimas antioxidantes endógenas, como a superóxido dismutase dependente de manganês (MnSOD), CAT, GPx e PRx (Bag e Bag, 2008).

Existem três isoformas conhecidas de SOD: cobre/zinco SOD citosólica (SOD1), cobre/zinco SOD extracelular (SOD3) e SOD mitocondrial (MnSOD ou SOD2) (Oberley e Oberley, 1997).

SOD3 ou SOD extracelular (EC) possui Cu e Zn junto ao seu sítio ativo e é a isoforma da SOD menos estudada dentre as três conhecidas. Nos humanos, 111 SNPs foram identificados para a SOD1, 190 para a SOD2 e 100 para a SOD3 (Crawford e cols., 2012).

Dentre essas três isoformas, SOD1 e SOD3 não demonstraram associações significativas com risco de câncer. Enquanto que SNPs de SOD2 têm sido amplamente relacionados com risco de câncer, sendo o polimorfismo Ala-9Val o mais investigado (Dansen e Wirtz, 2001; Cao e cols., 2014).

1.10 Polimorfismo da MnSOD e o Câncer

A enzima MnSOD, é um homotetrâmero que contém um íon de manganês por subunidade, está localizada na mitocôndria e é essencial para a sobrevivência celular de mamíferos. O gene da MnSOD é composto por cinco éxons e quatro íntrons, localizado no cromossomo 6q25.3 e tem sido identificado como um potencial gene supressor tumoral (Zhong, 1997).

O SNP mais estudado da MnSOD é Val16Ala (rs4880 ou rs1799725, rs1799725 incorporada ao rs4880), uma substituição de T por C no nucleotídeo 47, modificando a codificação do aminoácido valina (GTT, Val) para alanina (GCT, Ala) no 16º resíduo (16º aminoácido a partir do início do sinal de uma sequência de 24 aminoácidos). Esse resíduo é o 9º aminoácido a partir do local de clivagem, consequentemente, é usualmente designado como polimorfismo Ala9Val (Rosenblum e cols., 1996).

Uma vez que herdamos este polimorfismo, os possíveis genótipos que ocorrem nas populações humanas são: Ala/Ala, Ala/Val e Val/Val (Berto e cols., 2015).

Sugere-se que o polimorfismo altere a estrutura secundária da proteína e influencie o transporte mitocondrial da enzima MnSOD e a estabilidade de seu

mRNA. O polimorfismo interrompe a segmentação adequada da enzima do citosol para a matriz mitocondrial, onde atua no $O_2^{\cdot-}$ para converter a H_2O_2 . A troca de concentração de $O_2^{\cdot-}$ e H_2O_2 na mitocôndria modula os mecanismos moleculares de apoptose, adesão celular e proliferação celular (Sutton e cols., 2005; Arundhati e Niladri, 2008).

As investigações bioquímicas e moleculares sugeriram que o alelo A apresenta uma estrutura proteica α -hélice que se torna mais eficiente no transporte da MnSOD do citosol para o interior da mitocôndria e também na metabolização do $O_2^{\cdot-}$ em H_2O_2 . No entanto, tais resultados parecem contraditórios, uma vez que estudos sugerem associação do genótipo Ala/Ala com um risco maior de desenvolver câncer de próstata, mama, colón, esôfago e câncer de colo do útero (Sutton e cols., 2003; Taufer e cols., 2005; Murphy e cols., 2007; Tong e cols., 2009; Bica e cols., 2009; Wang e cols., 2009; Sun e cols., 2013; Chaiswing e cols., 2014).

Ambrosone e colaboradores estabeleceram que mulheres em pré-menopausa que fossem homozigotas para o alelo Ala tinham 4 vezes maior risco de câncer de mama quando comparadas com aqueles com o alelo Val. Uma meta-análise de 2009 mostrou que a MnSOD Ala/Ala contribui para um aumento significativo de risco de câncer de mama entre mulheres pré-menopausa com menos consumo de antioxidantes (Ambrosone e cols., 1999; Wang e cols., 2009).

A variante valina (Ala/Val ou Val/Val) apresenta uma estrutura β -pregueada, dificultando assim o seu transporte para o espaço mitocondrial e desta forma, reduzindo em 30-40% menos atividade antioxidante quando comparada ao alelo Ala. Ainda, o alelo Val pode estar associado com menor estabilidade do RNAm. Em contraste à hipótese de que o alelo Val possivelmente seja um alelo de maior risco, achados de um estudo entre fumantes pesados na Finlândia sugerem que o alelo Ala seja o alelo de alto risco para câncer de próstata. Os pacientes tiveram um aumento de 70% no risco para Ca de próstata e três vezes mais chance de desenvolver tumor de alto grau quando comparados com pacientes que possuísem genótipos Val/Val ou

Val/Ala (Rosenblum, 1996; Shimoda-Matsubayashi e cols., 1996; Sutton e cols., 2003; Woodson e cols., 2003; Sutton e cols., 2005; Barbisan e cols., 2014).

O genótipo MnSOD Ala/Ala tem sido associado com câncer de próstata em fumantes e em homens com menor capacidade antioxidante ou alta ingestão de ferro (Woodson e cols., 2003; Li H e cols., 2005; Kang e cols., 2007; Choi et al 2008; Mikhak e cols., 2008; Iguchi e cols., 2009). Entretanto, três recentes meta-análises demonstraram resultados conflitantes a respeito da associação entre o polimorfismo do gene MnSOD e o risco para câncer de próstata (Liwei e cols., 2009; Wang e cols., 2009; Mao e cols., 2010).

No *Physicians' Health Study* (PHS), homens com genótipo Ala/Ala foram também estabelecidos como mais suscetíveis ao câncer de próstata, mas apenas se tivesse *low plasma antioxidant status* (soma de licopeno plasmático, selênio e a-tocoferol). Em contraste, entre os homens com *high antioxidant status*, possuir o genótipo Ala/Ala foi associado com redução do risco (Li e cols., 2005).

Estudos em câncer de bexiga e MnSOD encontraram resultados conflitantes. Kucukgergin e colaboradores não encontraram associação significativa entre o polimorfismo Ala9Val da MnSOD e a suscetibilidade ao câncer de bexiga, enquanto que Hung e cols. descreveram que o genótipo Val/Val tem risco aumentado para câncer de bexiga (Hung e cols., 2004; Kucukgergin e cols., 2012).

Estes dados contraditórios encontrados na literatura, podem ser devido à forte influência de fatores ambientais, como alimentação, atividade física, bem como a origem étnica entre outros fatores (Bresciani e cols., 2013; Wang e cols., 2015).

Desta forma, o polimorfismo da enzima MnSOD ainda é um fator controverso relacionado à ocorrência de processos neoplásicos no organismo, e embora o estresse oxidativo seja considerado, atualmente, um importante fator de risco associado à carcinogênese, a modulação do mesmo pode ocorrer em

função de variantes genéticas, ambientais ou do sinergismo gene-ambiente (Bakalova e cols., 2013).

Tomando o conjunto de informações descritas até o presente momento, consideramos que investigar a associação do polimorfismo da MnSOD é de grande relevância. O estudo do estresse oxidativo e das variantes polimórficas da MnSOD conjuntamente à expressão dos biomarcadores IHQ associados a neoplasia vesical poderiam elucidar mecanismos da carcinogênese no urotélio, identificar grupos de risco e apontar fatores de risco para a recidiva e a progressão no carcinoma de bexiga.

1.11 Justificativa

A neoplasia de bexiga é a patologia urológica que mais onera o sistema de saúde porque a doença se caracteriza por recidiva e progressão. Estes pacientes necessitam várias ressecções das lesões vesicais, ressecções da bexiga, reconstrução cirúrgica do trato urinário, longas internações hospitalares, imunoterapia, radioterapia e quimioterapia no curso de suas doenças. Apesar dos recentes avanços no tratamento cirúrgico do câncer de bexiga como cistectomia e reconstrução do trato urinário, a carcinogênese é uma área controversa e com poucos achados eficazes. Biomarcadores e fatores de riscos associados a doença são temas promissores para estudos nessa área.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar o polimorfismo da enzima MnSOD e o uso de diferentes biomarcadores em paciente com carcinoma urotelial de bexiga.

2.2 Objetivos Secundários

- ☐ Revisar os atuais biomarcadores IHQ utilizados no diagnóstico e prognóstico de pacientes NMICB.
- ☐ Avaliar a expressão imunohistoquímica das proteínas p16, p21, p27, p53, pRb e Ki-67 em carcinomas uroteliais de bexiga
- ☐ Correlacionar as proteínas p16, p21, p27, p53, pRb e Ki-67 com as variáveis demográficas, estadiamento, tipo histológico e recorrência do carcinoma urotelial de bexiga.
- ☐ Determinar a frequência gênica e genotípica dos alelos da MnSOD Ala e Val na população em estudo.
- ☐ Estimar o risco do desenvolvimento de carcinoma urotelial de bexiga em relação aos genótipos investigados.
- ☐ Correlacionar o genótipo da MnSOD ao estadiamento, tipo histológico e recorrência do carcinoma urotelial de bexiga.
- ☐ Investigar as variáveis demográficas e associar o genótipo da MnSOD em pacientes com carcinoma urotelial de bexiga.

3 ARTIGOS CIENTÍFICOS

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Immunohistochemistry Biomarkers in Nonmuscle Invasive Bladder Cancer

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Abstract: Bladder cancer (BCa) is the most frequent urinary tract neoplasm. BCa results in significant mortality when the disease presents as muscle invasive. Around 75% to 80% of patients present with nonmuscle invasive bladder cancer (NMIBC), but recurrence and progression are significant issues, compelling current guidelines to recommend long-term surveillance. There is therefore an urgent and unmet need to identify and validate accurate biomarkers for the detection of disease recurrence to improve quality of life for the patients and reduce costs for health care providers, while maintaining or improving current outcomes. In this review, 38 publications on immunohistochemistry prognostic biomarkers, that were studied may be related in nonmuscle invasive bladder cancer, have been analyzed. The studies were organized according to the evaluated marker and their findings. It was demonstrated that the combination of independent complementary biomarkers could allow a more accurate prognosis than an isolated marker. Biomarkers, including p53, Ki-67, and CK20, with classic and prognostic factors with recurrence and novel markers such as EN2 may provide a more accurate prediction of outcome compared with any single marker, improving risk stratification and clinical management of patients with BCa.

Key Words: bladder cancer, biomarkers, immunohistochemistry, NMIBC

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Bladder Cancer (BCa) is the most frequent urinary tract neoplasm, the seventh most frequent in men and the 17th most frequent in women, with approximately 429,793 new cases diagnosed in 2012. The highest incidence rates are observed in developed countries, which

account for 59% of annual diagnoses. World mortality levels reached 165,068 deaths in 2012, is slightly higher than in the least developed countries.¹ Despite the higher prevalence in males, mortality is about 40% lower in this group, because the disease is 32% more likely to be diagnosed in the early stages, in relation to cases in females. This is due to the delay in diagnosis during confusion of initial symptoms such as hematuria and bladder irritation with urinary tract infection common among women.²

The incidence of BCa has decreased in some registries, which has been interpreted to reflect the decreased exposure to causative agents (mainly decreased smoking and better occupational hygiene).³ There is no uniform trend, however, as the age-standardized incidence rates declined in the United Kingdom, whereas the rates remained stable in white Americans in the same time period.⁴

The average age of initial diagnosis is 65 years.² Approximately 75% of patients with BCa feature a disease that is confined to the mucosa (stage Ta, carcinoma in situ) or submucosa (T1 phase). These categories are grouped as nonmuscle invasive bladder cancer (NMIBC). The NMIBC has a high prevalence due to low rates of progression and a long-term survival in many cases; patients with muscle invasive BCa (MIBC) are at greater risk of cancer-specific mortality due to invasion and metastases.⁵ The bladder neoplasms are derived from transitional epithelium in about 95% of cases, being the same tissue lining the urinary tract of the renal pelvis, ureter, and the proximal urethra. The other 5% correspond to the mesenchymal tumors, the most common is leiomyoma, which originates from muscle tissue.^{2,6}

The major risk factors that predispose individuals to BCa are well defined, namely genetic susceptibility, tobacco smoking, and occupational risk. The occupational risk is viewed as the second most important risk factor for BCa. It occurs when the individual is exposed to aromatic amines, aromatic polycyclic hydrocarbons, and chlorinated hydrocarbons—all used in industrial processing areas paint, dye, metal, and petroleum products.⁴ The exposure to ionizing radiation is connected with an increased risk of BCa. Ionizing radiation has many industrial, military, and medical imaging uses, as computerized tomography scans and nuclear medicine.⁵ Tobacco smoking is the strongest established risk factor for BCa in both men and women. Of the substances found in the smoke, the nitrosamines and

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β -naphthylamine, have known carcinogenic effects.⁷ A Brazilian case-control study performed by Moura et al,⁸ classified tobacco as a strong risk factor for cancers of the esophagus and bladder. Carcinogenesis associated with schistosomiasis, a chronic cystitis caused by recurrent infection with the parasitic trematode endemic in some parts of northern Africa (eg, Egypt), are mainly the squamous cell carcinoma type.⁴ Recent data from The Cancer Genome Atlas indicates that BCa harbor a large number of genomic mutations and that many of these are not related to previous smoking. This study revealed mutations in 32 genes, including multiple genes involved in cell cycle regulation, chromatin regulation, and kinase signaling pathways, as well as 9 genes not previously reported as significantly mutated in any cancer. Whole-genome and RNA sequencing identified recurrent in-frame activating *FGFR3-TACC3* fusions and expression or integration of several viruses (including HPV16) that are associated with gene inactivation.⁹

DIAGNOSIS

The initial detection of BCa is the association between urinary cytology and the cystoscopy examination. Although urinary cytology assesses the presence of cancer cells in the urine, cystoscopy allows full visual assessment of the bladder mucosa and of suspicious lesions.^{10,11} Cystoscopy, most commonly used due to its sensibility in low-grade and high-grade tumors, is invasive and relatively expensive, has a sensitivity of only 90% or so, thus limiting its use. Urine cytology has good sensitivity (28% to 100%) for detecting high-grade BCa and carcinoma in situ, but its sensitivity for detection of low-grade tumors is only 4% to 31%. Furthermore, the performance of cytology is dependent on the level of expertise of the cytopathologist.¹²

The gold standard diagnosis of BCa is the combined transurethral resection or tumor biopsy and histologic hematoxylin and eosin stain and immunohistochemistry (IHC) biomarkers. Biomarkers are substances that when measured can indicate whether a biological process is normal or pathologic, or whether there is a pharmacological response to any therapeutic intervention.¹³

Regarding terminology, a prognostic biomarker provides information about the patients overall cancer outcome, regardless of therapy. The presence or the absence of such a prognostic marker can be useful for the selection of patients for a certain treatment, but does not predict the response to this treatment. Prognostic biomarkers can be separated in 2 groups: biomarkers that give information on recurrence in patients who receive curative treatment and biomarkers that correlate with the duration of (progression free) survival in patients with metastatic disease. A biomarker with predictive value gives information on the effect of a therapeutic intervention in a patient. A predictive biomarker can also be a target for therapy.¹⁴

Surveillance of patients with history of NMIBC is a key area for the use of new markers. This is largely due to

the high prevalence and recurrence rate of the disease. Theoretically, in the surveillance setting, a marker could both reduce the number of cystoscopy and detect recurrence of progression earlier than traditional tests.¹⁵ In this review we focus on IHC-based studies on prognostic markers currently studied and some promising that may be related in NMIBC. As a complement, we suggest few biomarkers to improve the prognostication and predictive value in BCa patients.

METHODOLOGY

According to Medline, Cochrane Library, and PubMed database, an active virtual search has been carried out on “experimental studies on histopathological biomarkers for BCa,” published in the period between 2009 and 2014, without language restriction. Subject descriptors used in the research of articles were: BCa, NMIBC, markers, prognostic, and recurrence. More than 1200 publications were retrieved.

The eligibility criteria were assessed independently by 2 reviewers, and in case of disagreement, a third researcher was consulted. Sorting of articles was conducted from the analysis of their summaries to verify if they met the following criteria for eligibility: to present an outline of experimental study, suitable for the evaluation of a prognostic marker; having human beings as research subjects; having bladder tissue as biological sample; using IHC as the analytical method; objectify the investigation of prognostic markers; and include samples from patients with NMIBC or nonmuscular invasive urothelial carcinomas of the bladder. Studies restricted to squamous cell carcinomas, adenocarcinomas, MIBC, and small cell carcinomas, as well as studies whose purpose was to search for potential therapeutic targets, have been excluded.

This review summarizes the current existing tissue biomarkers in NMIBC patients, we have fully analyzed 38 publications on IHC biomarkers.

RESULTS

Patients with NMIBC require personalized treatment and regular cystoscopy surveillance that make this disease one of the most expensive malignancies in terms of lifetime dollars spent per patient. So, the identification of patients at risk for disease recurrence and/or progression remains challenging.¹⁶

Tissue-based biomarkers could help physicians provide individualized prognostications and allow risk-stratified clinical decision making regarding surgical and medical treatment, such as early radical cystectomy and neoadjuvant or adjuvant chemotherapy.¹⁵

Numerous candidate IHC tissue biomarkers for BCa have been identified and extensively studied. These markers include various pathways implicated in BCa, including cell cycle, apoptosis, proliferation, transcription, etc.¹⁵

Table 1 is divided into the biomarker pathway implicated, showing the distribution of the prognostic

TABLE 1. Immunohistochemistry Biomarkers in NMIBC

Biomarkers	References	N	Findings
Apoptosis			
Livina	Wang et al ¹⁷	138	Expression of 65.22% in NMIBC and 72.7% in T1. Significant association with less time of free overlife of recurrence
Survivin	Wang et al ¹⁷	138	Expression of 71.01% in NMIBC and 80% in T1. Significant association with less time of free over life of recurrence
Caspase-3	Wang et al ¹⁷	138	71.01% expression in NMIBC and 80% in T1. Significant association with shorter time to recurrence-free survival
<i>UHRF1</i>	Yang et al ¹⁸	118	High expression is correlated with recurrence ($P = 0.013$) and shorter survival time ($P = 0.0002$)
Cell adhesion			
P-cadherin	Wang et al ¹⁹	110	High expression correlated with tumor progression ($P = 0.031$), being an independent predictor of progression ($P = 0.042$), but not of recurrence ($P = 0.139$)
CD44v6	Klatte et al ²⁰	410	The expression is higher in NMIBC and to a lesser degree ($P < 0.001$). And its absence is related to a higher risk of recurrence
CD24	Liu et al ²¹	125	High expression associated with high grade ($P < 0.001$) and recurrence ($P < 0.001$).
TPBG	Xylinas et al ²²	384	Associated with staging ($P = 0.04$), but not with degree ($P = 0.49$), recurrence ($P = 0.76$) and cancer-specific mortality ($P = 0.32$)
Suppressor gene			
p53	Wang et al ²³	270	Semiquantitative evaluation of p53 correlates with degree ($P = 0.03$) and invasive conditions (0.035). Associated with Ki-67, has predictive value for recurrence in NMIBC ($P < 0.05$)
	Ogata et al ¹³	43	In superficial bladder cancer, showed strong association with degree ($P = 0.0001$) and progression ($P = 0.0076$), but not with recurrence ($P = 0.1715$)
	Maeng et al ²⁴	55	No significant relationship to degree, staging, or prediction of multivariate recurrence. Association with a lower recurrence-free survival in univariate analysis ($P = 0.017$)
p63	Wang et al ²³	270	Positive expression associated with level ($P = 0.021$), and staging ($P < 0.0001$), but not significant for predicting recurrence in NMIBC
Oncogene			
<i>UHRF1</i>	Unoki et al ²⁵	71	High expression in NMIBC ($P = 0.0063$) and in patients with high risk of progression ($P = 0.0350$)
IMP3	Xylinas et al ²²	384	Nonassociated with the degree ($P = 0.12$) or the staging ($P = 0.33$)
SKIP	Wang et al ²³	70	Expression is associated with high-grade tumor ($P = 0.001$) and recurrence ($P = 0.004$)
<i>AEG-1</i>	Yang et al ²⁶	102	High expression is associated with a high degree of tumor ($P = 0.002$), progression ($P = 0.028$), progression-free survival, and lower ($P = 0.0011$)
Transcription			
HDAC 1-3	Poyet et al ²⁷	174	HDAC 1-3 were not significantly predictive for progression
<i>EN2</i>	Morgan et al ²⁸	149	Immunoeexpression is 24 times greater in transitional cell carcinoma than in vesical normal tissue
HMGA2	Yang et al ²⁹	148	High expression associated with less recurrence-free survival ($P < 0.001$) and progression-free survival ($P = 0.0004$)
HMGB1	Yang et al ³⁰	164	High expression is associated with high-grade tumors ($P < 0.001$), with increased staging ($P = 0.001$), with less disease-free survival ($P < 0.001$) and lower overall survival ($P < 0.001$)
<i>CHD1L</i>	Tian et al ³¹	153	High expression is associated with high-grade tumors ($P = 0.005$) and the lowest survival rate of the patients ($P < 0.001$)
<i>BLCA-4</i>	Zhao et al ³²	325	High expression is associated with higher degree ($P < 0.001$)
Proliferation			
MAI	Mangrud et al ³³	193	With CI = 95%, showed 0.79 of sensitivity and specificity, and positive predictive value of 0.22 for progression
Ki-67	Mangrud et al ³³	193	With CI = 95%, showed 0.54 of sensitivity, 0.77 of specificity, and negative predictive value of 0.96 for progression
	Wang et al ²³	270	Its expression correlates with the degree ($P < 0.0001$), staging ($P < 0.0001$), tumor size ($P < 0.0001$). Combined with p53, revealed a recurrence predictive value in NMIBC ($P < 0.05$)
	Ogata et al ¹³	43	In superficial bladder cancer showed strong association with recurrence ($P = 0.0001$) and high-grade histologic ($P = 0.0001$)
	Maeng et al ²⁴	55	High expression associated with high grade ($P = 0.003$), high staging ($P < 0.001$), and frequency of recurrence (0.019)
	Bertz et al ³⁴	309	Expression correlated with progression ($P = 0.002$)
PPH3	Mangrud et al ³³	193	With CI = 95%, showed 0.54 of sensitivity, 0.77 of specificity, and negative predictive value of 0.96 for progression
HYAL-1	Kramer et al ³⁵	178	High expression in patients who presented progression or recurrence ($P < 0.001$)
Gli1	Sverrisson et al ³⁶	261	Expression is associated with less risk of relapse ($P < 0.05$)

TABLE 1. (continued)

Biomarkers	References	N	Findings
Cell growth <i>FGFR3</i>	Maeng et al ²⁴	55	Positivity is associated with a lesser degree ($P = 0.022$) and staging ($P = 0.011$), and absence of <i>FGFR3</i> -c linked to recurrence ($P = 0.027$)
Immunomarker <i>COX-2</i>	Tadin et al ³⁷	127	The expression profile demonstrates it as a good predictor for recurrence ($P = 0.004$)
Miscellaneous <i>CK20</i>	Bertz et al ³⁴ Ogata et al ¹³	309 43	Expression correlated with recurrence in multivariate analysis ($P = 0.014$) In superficial BCa, showed association with recurrence ($P = 0.0166$) and high histologic degree ($P = 0.0002$)
<i>MAGE-A</i>	Xylinas et al ²²	384	Nonassociated with the degree ($P = 0.29$) or the staging ($P = 0.16$)
<i>HA-1</i>	Kramer et al ³⁵	178	Expression is associated with muscle invasion ($P < 0.001$)
<i>Usp28</i>	Guo et al ³⁸	186	High expression is associated with recurrence ($P = 0.0051$)

NMIBC indicates nonmuscle invasive bladder cancer.

markers according their findings, the result obtained, the year of publication and its authors.

DISCUSSION

This utility and importance of biomarkers has been recognized and increasingly studied. However, despite the increase in interest and investment in them, few biomarkers are used in routine clinical practice, which makes further studies necessary, aimed at proving a biomarker's prognostic and predictive value, alone or combined, and its implementation in clinical practice.¹³

Some studies have been widely carried out correlating IHC markers, including p53, Ki-67, and CK20, with classic and prognostic factors with recurrence.^{13,23,24,33,34} Others studies investigated novel markers such as EN2.²⁸ These potential biomarkers either alone or in combination may help improve risk stratification and clinical management of patients with BCa.

Thereafter we have compiled 4 markers considered promising by our group, because they were cited by several articles and/or showed potential in prognostic and predictive value in studies with BCa and other cancers.

p53

p53 is a protein expressed from the gene TP53, one of the most studied tumor suppressor genes, which is involved in the maintenance of genome stability, in response to genotoxic stress and cell cycle regulation. There are several human cancers to which a mutation or gene overexpression in p53 has been linked.^{23,24} In BCa a well-performed retrospective study supports that the nuclear accumulation of p53 is prognostic of outcome, especially in patients treated with radical cystectomy.³⁹ In NMIBC, a study of 80 consecutive patients with pT1N0 BCa who underwent radical cystectomy, 25% had altered p53 expression. These patients had an increased risk of disease recurrence, disease progression, and cancer-specific mortality.^{40,41} In addition, some studies reported that altered p53 expression was predictive of response to bacillus Calmette-Guerin treatment in patients with NMIBC.^{42–44}

Tumor protein p53 might be related to recurrence, Shariat et al⁴⁵ has shown that p53 status improved the accuracy of clinicopathologic parameters for prediction of disease recurrence and cancer-specific mortality by a statistically and prognostically significant margin.

Other studies have investigated the significance of p53 for prediction of recurrence when associated to another marker. Wang et al,²³ for example, has shown there is significance, at least for NMIBC, combining the p53 to Ki-67 ($P < 0.05$). Thus, the potential use of p53 as a marker of recurrence may be in combining it with other markers to obtain statistical significance.

Ki-67

Ki-67 protein is strongly related to cell proliferation, being expressed in proliferating cells along the phases G1, S, G2, and M, and providing a reliable cell proliferation index.²⁴ Different studies have demonstrated an association between a high rate of staining for Ki-67 and a prognosis of unfavorable disease.^{24,33,34} Its expression is commonly assessed by IHC using the MIB1 antibody. The prognostic and predictive values of the Ki-67/MIB1 proliferation marker in BCa have been extensively studied.^{46,47} In NMIBC, MIB1 has been shown to be significantly related to recurrence, progression-free survival, and disease-specific survival according to Shariat et al.⁴⁰ Although others failed to confirm an association with overall disease-free survival.^{48–50}

As a proliferation marker, significant number of studies agree that Ki-67 increases its expression significantly in higher grade tumors and higher stage.^{13,23,24} Wang et al²³ found a correlation between the expression of Ki-67 and larger tumors and multiple. These results indicate that the expression of Ki-67 is associated positively with the invasion and the level of malignancy of BCa.

However, in contrast with the line of the authors on the relationship of Ki-67 in the grade and the staging in the BCa, the predictive value of recurrence remains controversial. Ogata et al,¹³ found a significant association between Ki-67 and recurrence ($P = 0.0001$), as well as

Maeng et al²⁴ noted that the high reactivity of the Ki-67 was related to recurrence in 2 years ($P = 0.019$). Nevertheless, Maeng et al²⁴ found no prognostic value independent of the Ki-67 for tumor recurrence, and Wang et al²³ obtained a significant correlation between the rate of recurrence in noninvasive tumors and the expression of Ki-67 only when in univariate analysis.^{23,24} Thus, it is necessary to carry out a greater number of studies to examine the potential of Ki-67 in the prognosis of tumor recurrence, perhaps this disagreement between the results may be due to the different classification systems, employees or cutting points using different monoclonal antibodies.¹³

Cytokeratin 20 (CK20)

CK20 is a protein family of cytokeratins found in intermediate and superficial cell cytoskeleton of normal bladder urothelium. Its abnormal expression has been detected in the BCa and seems to be associated with recurrence of the tumor, especially in noninvasive urothelial carcinomas (Ta/T1).^{13,34} Studies such as Ogata et al¹³ have revealed statistical association between anomalous CK20 expression and relapses ($P = 0.0166$). Similarly, it seems consensus among the authors that the CK20 is correlated with the histologic grade of the tumor. The only divergent aspect to this protein appears as regards to its predictive value in the progression. Ogata and colleagues did not find any significance to relate the CK20 with histologic progression ($P = 0.4079$) or clinical progression ($P = 0.1591$), whereas studies have associated the CK20 to Ki-67, which in coexpression were related to minor progression-free survival and the biggest degree and tumor staging, which would explain the role of CK20 in a bad prognosis. The coexpression of 2 proteins can then play an important role in the progression of BCa.^{13,34}

EN2

EN2 is a transcription factor recently described as a potential tumor marker. Despite having only a study relating the EN2 with the BCa, it has proved its value while oncogenic biomarker studies with other carcinomas, like prostate cancer.⁵¹ Morgan et al²⁸ showed that this protein presents cancer-specific expression in tumors of BCa, being sensitive even when early in the disease. In this same study, was not found any staining in IHC for vesical normal tissue, in contrast to a 24 times greater for fabric with urothelial carcinoma. Also noted that the higher histologic grade, was smaller was the expression on EN2. Such results confirm the potential of EN2 while marker for the BCa, which can have its consolidated paper coupled the new studies.

CONCLUSIONS

BCa is a heterogeneous neoplasia that presents high probability of recurrence and progression (only 15% progress to invade and metastasis). Thus, it is of the utmost importance that their identification and stratification clinicopathological be made as accurately as possible, allowing a proper management of the disease. Biomolecular markers

act, in that context, to increase the accuracy of prognoses, and consequently allow for individualized treatment and more effective. However, because the molecular abnormalities of BCa are highly complex as demonstrated by recent TCGA data, it is unlikely that a single marker is capable of accurately segregating tumors of similar clinicopathologic phenotypes into precise prognostic categories. Thus, as it has been demonstrated by various studies, the combination of independent complementary biomarkers could allow a diagnosis or a more accurate prognosis than an isolated marker, may provide a more accurate prediction of outcome compared with any single marker, making the identification of biomarkers panels an important topic for research. Future investigations should focus on promising biomarker combinations that encompass a variety of different pathways to increase the predictive value and possibility for targeted therapy.

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3.2 Artigo II submetido a revista Pathology and Oncoly Research: “Cell cycle markers in the evaluation of bladder cancer”.

Cell cycle markers in the evaluation of bladder cancer

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Abstract

Objectives: Bladder cancer (BC) is a heterogeneous neoplasia characterized by a high number of recurrences. Standardized clinical and morphological parameters are not always sufficient to predict individual tumor behavior. The aim of this study was to evaluate the expression of cell cycle regulators proteins as potential adjuvant in prognosis and monitoring of this disease. **Materials and Methods:** Block paraffin samples from patients with urothelial bladder carcinoma treated by transurethral resection (TUR) were collected to immunohistochemistry analysis for proteins p16, p21, p27, p53, pRb and Ki-67. Chi-square, logistic regression and Kaplan-Meier curve were used to analyze the prognostic value of these markers. **Results:** Of the 93 patients included in the study, the main categories of staging observed were T1 (53%) and Ta (29%), and the distribution between tumor grades was 58% of patients with low grade and 42% of patients with high grade. The expressions of p16, p21, p27, p53, pRb and Ki-67 were altered in 31%, 42%, 60%, 91%, 27% and 56% of patients, respectively. The immunohistochemical expression of Ki-67 was associated with tumor histological grade ($p = 0.016$), and expression of pRb with recurrence-free survival ($p = 0.035$), but no isolated marker was significant associated with recurrence and progression in multivariate analysis. More than two markers abnormally expressed were associated with presence of recurrence ($p = 0.005$) and lower recurrence-free survival ($p = 0.004$). **Conclusions:** Our panel marker has important prognostic value for BC, especially when more than two have altered expression predicting good clinical recurrence implication.

Keywords: Bladder cancer; Cell cycle markers; Prognosis; Immunohistochemistry

Introduction

Bladder cancer (BC) is a heterogeneous disease highly associated with smoking. It is the most frequent urinary tract neoplasia, with approximately 430,000 new cases diagnosed in 2012 [1,2]. Ninety percent of BC are urothelial carcinomas that can be, according to the TNM staging, as non-muscle invasive (Ta, Tcis, T1) or muscle invasive (T2, T3, T4) [3,4]. The majority of BC (75%) are non-muscle invasive. These tumors can recidivate in 60-70% or progress in 20-30% of the cases [5,6].

Cystoscopy, urinary cytology and transurethral resection (TUR) are utilized to establish the diagnosis and follow up in BC [6,7]. Histological grade and TNM staging are important prognostic factors for aggressiveness in BC [6]. However, due to BC heterogeneity, there is no definitive parameter to predict the behavior and the prognosis of BC [8,9].

For this reason, several markers have been investigated to complement standart cytopathology and histopathological examination. Cell cycle control and proliferation related proteins have been investigated as auxiliary in BC identification and prognostic factors in BC treatment [4,9-13].

The aim of this study was to identify an immunohistochemical panel of cell cycle regulatory proteins (p16, p21, p27, p53, pRb and Ki67) in a retrospective cohort of BC patients and to predict its association with pathological parameters (grade and muscle invasion), recurrence and progression.

Materials and Methods

Ethical Considerations

This study meets The Code of Ethics of the World Medical Association (Declaration of Helsinki), having been approved by the Ethical Committee of the Hospital. Patients received clear explanation about the study aims, data secrecy and

confidentiality and the volunteer character of the participation upon Informed Consent (IC) signature.

Study Population

The study population was composed of a retrospective cohort of patients diagnosed with urothelial bladder carcinoma, after transurethral resection (TUR). During the period of December 2000 and May 2014, patients from Santa Rita Hospital, ISCMPA/BR that meet the following criteria; be older than 18 years and have no evidence of associated cancer were selected. Study population was selected based on histopathological reports from Laboratório de Patologia do Hospital Santa Rita.

Data Collection

Demographic data was collected using a sociodemographic questionnaire. We had access of patients' medical chart including exams and histopathological reports. Paraffin blocks was collected and slide staining for hematoxylin-eosin (H&E) and for immunohistochemistry (IHC) was performed. H&E slides were reviewed by a Pathologist for diagnosis confirmation, following the International Union Against Cancer 2009 classification for TNM staging and World Health Organization/ International Society of Urological Pathology 2004 classification for grade.

Immunohistochemistry

Immunohistochemical staining was performed for p16, p21, p27, p53, pRb and Ki67 proteins, according to the peroxidase method. The slices, 4µm thickness, were deparaffinized and rehydrated according to standard protocols [14]. Antigen retrieval was then performed using tris-EDTA buffer (ethylenediaminetetraacetic acid; pH 9.0) for Ki-67 and p53 markers, and Sodium Citrate (pH 6.0) for p16, p21, p27 and pRb markers, for 40 minutes in a waterbath at 95-98°C. After heating, the slices were cooled at room temperature for 20 minutes, followed by blocking of the endogenous peroxidase activity

through immersion in water with 5% H₂O₂ (3 times of 10 minutes each). In sequence, slides were washed with phosphate buffered saline (PBS) two times, and incubated in a solution to block unspecific binding (bovine serum albumin 1%, for 1 hour) [14].

The primary antibodies used were as follows: p16 (clone 6H12, Novocastra; Newcastle; UK; dilution 1:40), p21 (clone DCS-60.2, Neomarkers; Fremont; USA; dilution 1:100), p27 (clone 5X53G8, Dako; Cambridge; UK; dilution 1:100), p53 (clone DO-7, Dako; Cambridge; UK; dilution 1:100), Ki-67 (clone MIB-1, Dako; Cambridge; UK; dilution 1:200) e pRb (clone Rb1, Zymed; San Francisco; USA; dilution 1:200).

The primary antibodies were applied; the slides were incubated in a dark humid chamber for 60 minutes at room temperature, and then remained in the fridge, at 4°C overnight. After this procedure, the slides were left at room temperature for 1 hour, and then washed with PBS. Slides were incubated with secondary antibody (Dako advance TM HRP link) for 1 hour, then washed again with PBS and incubated with tertiary antibody (Dako Advance TM HRP enzyme) for 1 hour. The antigen-antibody complex was visualized with diaminobenzidine tetrahydrochloride (DAB) and contrasted with hematoxylin [15].

Positive control staining for p53, p21 and p27 was performed on breast slices; hypophysis slices for p16; tonsil slices for Ki-67 and pRb. For the negative control, the primary antibodies were substituted with bovine serum albumin 1%.

Scoring

Five representatives fields presenting good marker expression were captured in each slide with an Olympus BX51 optical microscope equipped with a DP72 camera and DP2-BSW software (OlympusTM; Tokyo; Japan). Each hotspot underwent manual count of nuclear positivity in 200 cells; two independent evaluators performed the count with Image-Pro Plus 6.3 software. Absence of expression was considered only when no

immunohistochemical reaction could be observed. If discordance was greater than 20% between evaluators, a third researcher was consulted.

Nuclear immunoreactivity was considered altered when samples demonstrated expression $\geq 10\%$ for p53 and Ki67; 0% or $>50\%$ for pRb and p16; $<10\%$ for p21; and $<30\%$ for p27, according to the literature [8, 16, 17].

Statistical Analysis

Data were plotted, processed and analyzed with software SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Chi-square and Fisher's Exact Test were utilized to verify association between clinical parameters and protein expression and smoking. For the multivariate analysis logistic regression was utilized. The Kaplan-Meier method was utilized to evaluate patients' time free of recurrence. The differences were considered statistically significant to a level of significance of 5% ($p < 0.05$).

Results

Sample Characterization

A total of 108 patients were selected to participate in the study, however 15 were excluded because the paraffin block was not available. The final study sample consisted of 93 patients whose clinical-pathological characteristics are summarized in Table 1.

Marker Expression and Clinical-pathological Parameters

As shown in Table 2, Ki-67 expression was significantly associated with high grade BC ($p = 0.016$), but not with the muscle invasion. Association with clinical-pathological parameters was not observed for all other proteins. Figure 1 shows the expression pattern of markers in high and low histological grade of BC.

No isolated marker showed significant association with recurrence and progression (Table 3). However, when we evaluated the number of altered markers, a relation with recurrence was observed ($p = 0.023$). Multivariate analysis demonstrated

association with recurrence cases and altered expression in two or more markers ($p=0.005$).

Recurrence-free survival

Estimation of recurrence-free survival in 10 years related to markers expression, are demonstrated in Figure 2. The isolated proteins p16, p21, p27, p53 did not show significant relation to recurrence-free survival, but the altered expression in Ki-67 presented a tendency in lower recurrence-free survival in BC patients ($p=0.059$). In the multivariate analysis by logistic regression, no protein was revealed as a significant recurrence predictor.

Abnormal expression of pRb was clearly related to lower recurrence-free survival compared to pRB normal expression in BC patients ($p=0.035$). Patients who had one or two markers altered had higher recurrence-free survival than the patients with more than two markers abnormally expressed ($p=0.004$).

Smoking as a Risk Factor

Smoking status was present in 92% among the study population. Therefore, patients who had contact with cigarettes, as direct smoking or second-hand smoke, did not display significant difference from non-smokers in terms of grade, invasion, recurrence or disease progression in our study.

Discussion

BC is a heterogeneous neoplasia that presents high probability of recurrence and progression, showing different rates of metastasis and mortality, depending on tumor grade and staging. As reported by many authors and guidelines, BC occurs mostly in men, with a three times higher proportion than women [3,6,18,19]. Our study sample had a ratio of 2.57 men for each woman, being very close to the population estimate. In the

same way, the mean age at diagnosis was 63 years, showing accordance with preexisting data (60 – 70 years) [8, 16, 20].

Regarding bladder tumor grade and TNM classification, it is known that, low grade and pT1 cases are most common than others categories [6]. In this study, we observed that 58% of the patients had a low grade diagnosis and the most prevalent staging was pT1 (53% of the patients). BC grade and stage are the main prognostic indicators used in medical clinical practice [6]. However, due to high biological heterogeneity of BC, sometimes these parameters become insufficient to safely predict the aggressive tumor behavior [8,11,16].

In this context, several studies [8,16,17,21-24] have been performed associating immunohistochemical markers with grade, staging and recurrence. These markers include proteins responsible for cell cycle control and proliferation, corroborating the hypothesis that their expression levels can be explored as prognostic factors, either alone or in immunohistochemical panels [17].

In our study, the isolated analysis of p53, p16, p21, p27 and pRb proteins did not show significant association with grade, invasion, recurrence or progression. These results agree with Olsson *et al.* (2012) [17], which also did not reveal association in these parameters and the proteins p16, p53, p21 and pRb. The study by Lee *et al.* (2010) [25] obtained a significant association in the invasion of the BC and the altered expression of pRb and p53. An explanation for the divergence of data can be the heterogeneity of the populations studied, as well as the criteria used for marker classification. For p16, Olsson *et al.* (2012) [17] used a cutoff of 0% or >50%, while Lee *et al.* (2010) [25] and Kruger *et al.* (2005) [26] considered 0% or >76% and <10% respectively.

Studies with Ki-67 proliferation index have shown a significant association with grade, staging and recurrence, demonstrating its strongly relation with tumor

aggressiveness. [13, 21, 22, 27]. Our results confirm these findings, presenting significant association with high tumor grade and altered expression of Ki-67 ($p = 0.016$). The association of this protein with recurrence actually remains in conflict between studies. Weihong et al. (2014) [27] reported a significant association between altered Ki-67 and recurrence in both univariate and multivariate analysis. Other studies only obtained correlation for univariate analysis [21,22]. Most authors, including us, used a cutoff criteria for determination of altered proliferation equal to $\geq 10\%$ [16], however others considered a cutoff $\geq 20\%$ [8,22].

With the Kaplan-Meier curve, we demonstrated an association of pRb protein with lower recurrence-free survival in 10 years ($p = 0.035$ and $p = 0.059$ respectively), and a further tendency association for Ki-67 ($p = 0.059$). Weihong *et al.* (2014) [27] found an association between Ki-67 expression and the recurrence-free survival ($p < 0.0001$), when analyzing only non-muscle invasive BC.

As important finding in our study, we demonstrated that more than two cell cycle altered markers are associated with recurrence and lower recurrence-free survival in BC patients. These data confirm previous findings by Lotan *et al.* (2013) [16] who demonstrated significance in the number of altered biomarkers and predicted disease recurrence ($p = 0.004$). These results corroborate with the hypothesis that the combination of markers has a greater prognosis capacity than the isolated markers.

Cell cycle and proliferation regulatory proteins are potentially able to improve the prognosis ability of clinical-pathological parameters currently used, however, due to high biological and clinical heterogeneity of BC it is unlikely that a single marker is capable to predict precise prognostic categories. Therefore, an important finding for determination of recurrence using our marker panel seems to be how many of cell cycle markers have altered expression, specially more than two. However, more studies are

needed in order to increase the reproducibility of these results, given its important implications in the clinical management of patients with BC.

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Table 1. Clinical and pathologic characteristics of patients.

	n = 93
Median follow-up period, mo, median (IQR)	27 (13-48)
Age at diagnosis, yr, median (IQR)	63 (56-69)
Gender, no. (%)	
Male	67 (72)
Female	26 (28)
Histological Grade, no. (%)	
Low	54 (58)
High	39 (42)
Tumor Stage, no. (%)	
pTa	27 (29)
pTis	0
pT1	49 (53)
pT2	13 (14)
pT3	3 (3)
pT4	1 (1)
Recurrence, no. (%)	
Yes	23 (25)
No	70 (75)
Progression, no. (%)	
Yes	3 (3)
No	90 (97)
Smoking Status, no. (%)	
Active	82 (88)
Passive	4 (4)
No	7 (8)
IQR = Interquartile range	

Table 2. Nuclear immunoreactivity related to tumor grade and muscle invasion.

	Total	Grade			Muscle Invasion		
		Low	High	<i>P</i> value	Non-invasive	Invasive	<i>P</i> value
p53, no. (%)							
Normal	8 (8,6)	6 (11,1)	2 (5,1)	0,461	6 (7,9)	2 (11,8)	0,635
Abnormal	85 (91,4)	48 (88,9)	37 (94,9)		70 (92,1)	15 (88,2)	
p16, no. (%)							
Normal	64 (68,8)	37 (68,5)	27(69,2)	1,000	53 (69,7)	11 (64,7)	0,908
Abnormal	29 (31,2)	17 (31,5)	12 (30,2)		23 (30,3)	6 (35,3)	
p21, no. (%)							
Normal	54 (58,1)	33 (61,1)	21 (53,8)	0,626	46 (60,5)	8 (47,0)	0,456
Abnormal	39 (41,9)	21 (38,9)	18 (46,2)		30 (39,5)	9 (53,0)	
p27, no. (%)							
Normal	37 (39,8)	22 (40,7)	15 (38,5)	0,994	29 (38,1)	8 (47,0)	0,686
Abnormal	56 (60,2)	32 (59,3)	24 (61,5)		47 (61,9)	9 (53,0)	
pRb, no. (%)							
Normal	68 (73,1)	40 (74,0)	28 (71,8)	0,994	58 (76,3)	10 (58,8)	0,243
Abnormal	25 (26,9)	14 (36,0)	11 (28,2)		18 (23,7)	7 (41,2)	
Ki-67, no. (%)							
Normal	41 (44,1)	30 (55,6)	11 (28,2)	0,016	34 (44,7)	7 (41,2)	1,000
Abnormal	52 (55,9)	24 (44,4)	28 (71,8)		42 (55,3)	10 (58,8)	

Table 3. Markers alteration according to recurrence and progression

	Total	Recurrence			Progression		
		Yes	No	<i>P</i> value	Yes	No	<i>P</i> value
p53, no. (%)							
Normal	8 (8,6)	2 (8,7)	6 (8,6)	1,000	0	8 (8,9)	1,000
Abnormal	85 (91,4)	21 (91,3)	64 (91,4)		3 (100)	82 (91,1)	
p16, no. (%)							
Normal	64 (68,8)	14 (60,9)	50 (71,4)	0,491	1 (33,3)	36 (40,0)	0,228
Abnormal	29 (31,2)	9 (39,1)	20 (28,6)		2 (66,7)	27 (60,0)	
p21, no. (%)							
Normal	54 (58,1)	11 (47,8)	43 (61,4)	0,366	1 (33,3)	53 (58,9)	0,57
Abnormal	39 (41,9)	12 (52,2)	27 (38,6)		2 (66,7)	37 (41,1)	
p27, no. (%)							
Normal	37 (39,8)	8 (34,8)	29 (41,4)	0,749	2 (66,7)	35 (38,9)	0,561
Abnormal	56 (60,2)	15 (65,2)	41 (58,6)		1 (33,3)	55 (61,1)	
pRb, no. (%)							
Normal	68 (73,1)	14 (60,9)	54 (77,1)	0,209	3 (100)	65 (72,2)	0,561
Abnormal	25 (26,9)	9 (39,1)	16 (22,9)		0	25 (27,8)	
Ki-67, no. (%)							
Normal	41 (44,1)	8 (34,8)	33 (47,1)	0,427	0	41 (45,6)	0,252
Abnormal	52 (55,9)	15 (65,2)	37 (52,9)		3 (100)	49 (54,4)	
Altered markers no. (%)							
0	0	0	0	0,023	0	0	0,399
1	5 (5,4)	0	5 (7,1)		0	5 (5,6)	
2	25 (26,9)	2 (8,7)	23 (32,9)		0	25 (27,7)	
3	35 (37,6)	13 (56,5)	22 (31,4)		2 (66,7)	33 (36,7)	
4	18 (19,4)	3 (13,1)	15 (21,4)		0	18 (20,0)	
5	7 (7,5)	4 (17,4)	3 (4,3)		1 (33,3)	6 (6,7)	
6	3 (3,2)	1 (4,3)	2 (2,9)		0	3 (3,3)	
Altered markers no. (%)							
≤2	30 (32,3)	2 (8,7)	28 (40,0)	0,005	0	30 (33,3)	0,224
>2	63 (67,7)	21 (91,3)	42 (60,0)		3 (100)	60 (66,7)	

Nuclear immunoreactivity was considered altered when samples demonstrated expression $\geq 10\%$ for p53 and Ki67; 0% or $>50\%$ for pRb and p16; $<10\%$ for p21; and $<30\%$ for p27.

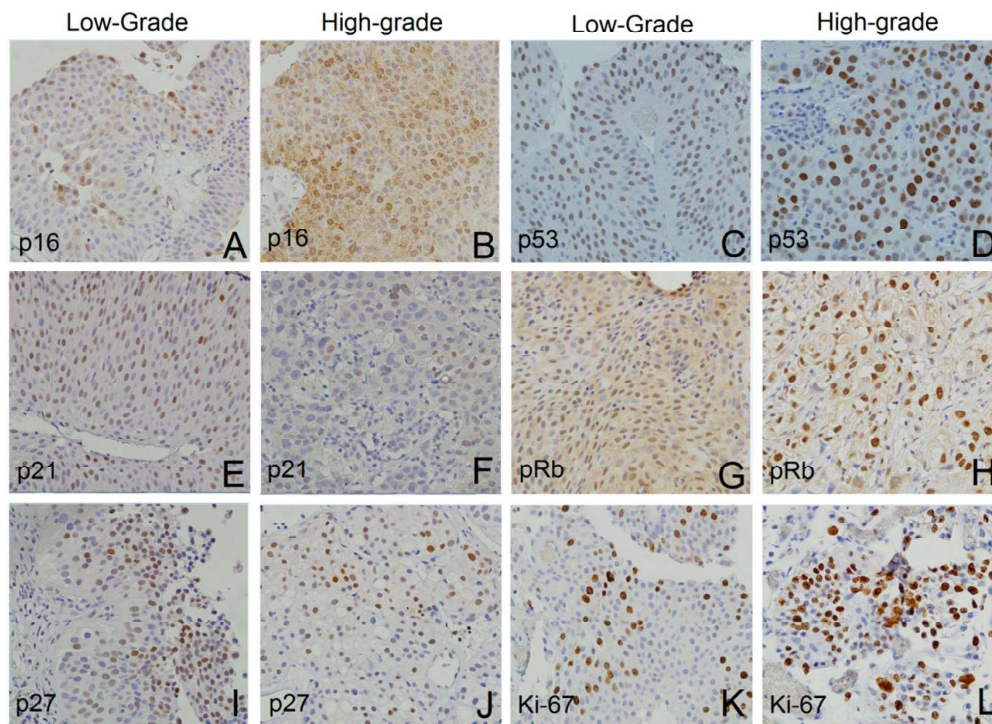


Figure 1. Immunohistochemical expression. Low and High-grade for p16 (A,B), p53 (C,D), p21 (E,F), pRb (G,H), p27 (I,J) and Ki-67 (K,L). (x200).

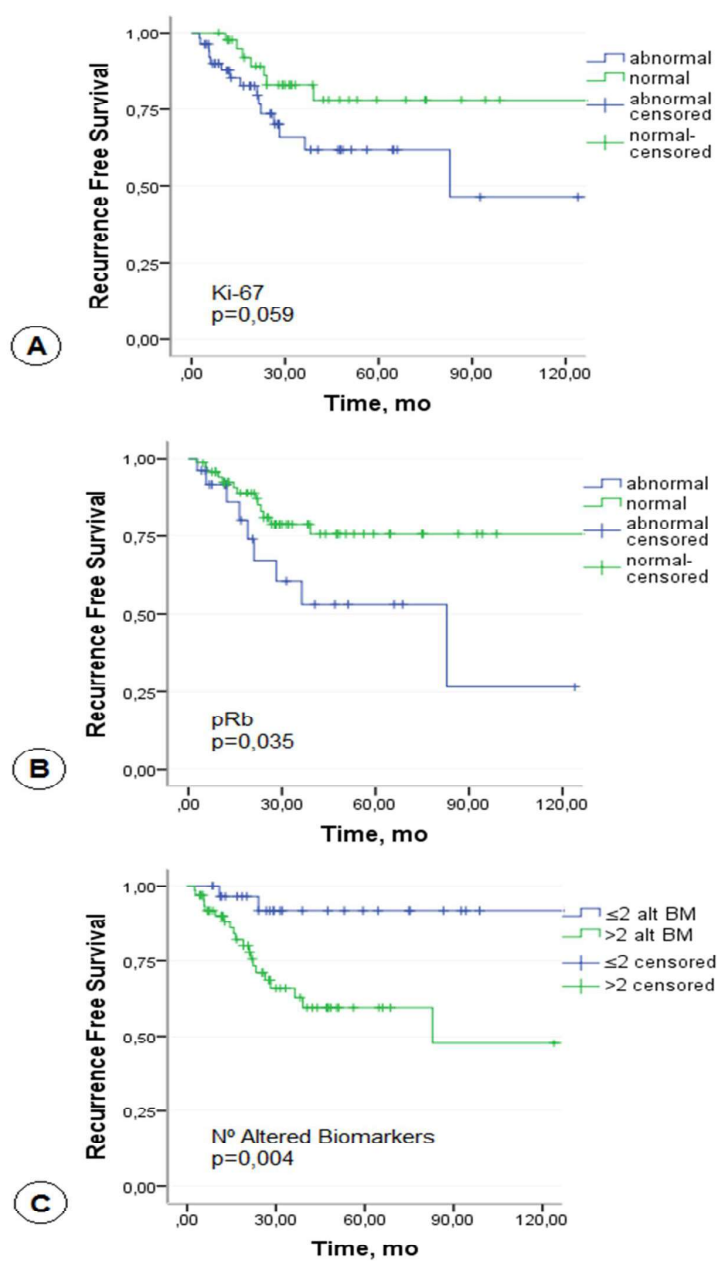


Figure 2. Kaplan-Meier analysis of recurrence-free survival for Ki-67 (A), pRb (B) and number of altered biomarkers (C). alt.=altered; BM = biomarker.

3.3 Artigo III submetido a revista Journal of Biomedical Science: “Association between bladder cancer risk and genetic variants of MnSOD enzyme”.

Association between bladder cancer risk and genetic variants of MnSOD enzyme.

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Abstract

Background: Bladder cancer (BCa) is the 7th most commonly diagnosed cancer in the male population worldwide, while it drops to 11th when both genders are considered. It is believed that individual susceptibility to BCa depends on an association of environmental and genetic risk factors, which could increase chronically reactive oxygen species (ROS) causing oxidative stress. This is the case of some occupational exposures, oxidative dietary factors and cigarette smoking. ROS are present in the composition of cigarette smoke, a well known carcinogenic factor strongly associated with BCa risk. On the other hand, some genetic variations can cause oxidative imbalance increasing the susceptibility of oxidative stress by their carriers. This is the case of Val16Ala single nucleotide polymorphism (SNP) that occur in the superoxide dismutase manganese dependent enzyme (MnSOD). This polymorphism has been associated with risk of several cancer types. Therefore, we investigated the association between BCa risk and the genetic variants of Val16Ala-MnSOD, an antioxidant enzyme, as well as its potential relationship with grade, stage and recurrence of the disease.

Methods: Initially a case-control study was conducted in order to evaluate the association between Val16Ala-MnSOD SNP and BCa. 357 subjects were enrolled (110 cases and 247 controls); all subjects had 5 mL of total peripheral blood collected. Genomic DNA fragments containing the MnSOD Ala-9Val polymorphism in the human SOD2 gene sequence were amplified by tetraplex PCR. A 36 months follow up of BCa patients was performed to evaluate the association between the polymorphism with cancer recurrence and mortality.

Results: BCa risk was found to be significantly associated with men patients ($P = 0.009$). We found an important association with Ala/Ala genotype and risk of BCa ($P = 0.001$), when compared with Val-carrying patients. No association between Ala-9Val polymorphism and BCa was observed in smokers or non-smokers in this study and this polymorphism may not play an important role in BCa grade. Staging T1 and low grade cancers were the most commonly cases observed in this study, but was not observed significant association with Val16Ala-MnSOD SNP and BCa aggressiveness, recurrence and mortality.

Conclusions: This study suggested that the Val16Ala-MnSOD SNP could be considered as a risk factor for BCa.

Keywords: Bladder cancer, oxidative stress, polymorphism, MnSOD.

Background

Bladder cancer (BCa) is the 7th most commonly diagnosed cancer in the male population worldwide, while it drops to 11th when both genders are considered. BCa had approximately 429,793 new cases diagnosed in 2012 around the world and 165,068 deaths in the same year [1]. About 75% of BCa cases present as non-muscle-invasive bladder cancer (NMIBC), with long-term survival but frequent recurrences [2–3]. Patients with muscle-invasive bladder cancer (MIBC) are at greater risk of cancer-specific mortality due to invasion and metastases [4].

It is believed that individual susceptibility to BCa depends on an association of environmental and genetic risk factors, such as occupational exposure, dietary factors and cigarette smoking [5–6]. Besides this, cigarette smoking is considered a major risk factor for both sexes, and increases relative risk two to four times when smokers are compared with non-smokers [7]. Cigarette smoke contains chemical carcinogens and reactive oxygen species (ROS) in its composition [8], which play a role in interacting with dietary oxidant intake and antioxidant status to lead to cancer development [9]. ROS are constantly generated by aerobic organisms as a result of normal metabolism. However, an increased level of ROS causes oxidative stress and creates a potentially toxic environment for cells, inducing DNA damage (DNA strand breaks, base modifications and base-free sites), protein oxidation and lipid peroxidation [10].

Among antioxidant enzyme systems against oxidative stress, MnSOD is an ROS catalyst dismutase (transforming O_2^- into H_2O_2), and is located in a major site of ROS production – inside the mitochondria [11].

The MnSOD gene has a single nucleotide polymorphism (SNP) at codon 16 (rs4880) which encodes for either alanine (Ala) or valine (Val) at the -9 position in the mitochondrial targeting sequence (Val16Ala-MnSOD SNP) [12]. It has been described that this polymorphism alters the secondary structure of the protein and influences mitochondrial transport of the MnSOD enzyme and its mRNA stability [13]. The Ala MnSOD variant, with an α -helix structure, is easily imported and generates high mitochondrial activity, whereas the Val MnSOD variant, with a partial β -sheet structure, has a decreased transport rate and results in low mitochondrial activity [14].

The variation in genes involved in the metabolism of ROS is considered to be a potential risk factor in carcinogenesis [15]; therefore, we have investigated the association between BCa risk and the genetic variants of Val16Ala-MnSOD SNP as well as its potential relationship with grade, stage and recurrence of the disease.

Methods

Study population

A prospective case-control study was conducted in order to evaluate the association between Val16Ala-MnSOD SNP and BCa. During the period of 2014 to 2015 a total of 110 bladder cancer patients from Santa Rita Hospital (Santa Casa de Misericórdia de Porto Alegre Medical Complex, Brazil - ISCMPA) and 247 healthy subjects were included. All controls came from an academic community in the south of Brazil and were paired by sex and age with the cases. Histopathological information about the primary tumour was collected from pathology reports and patients' medical records. All subjects were questioned

about their smoking status. The study was approved by the ISCMPA ethics committee (226.953) and written informed consent was obtained from all subjects included in the study.

Individuals who had never smoked or had smoked for < 1 year were considered as non-smokers, and individuals who currently smoked or gave up smoking at the time of diagnosis were categorized as smokers. None of the patients had a history of any kind of tumour other than BCa.

BCa was diagnosed histologically with specimens obtained by biopsy or surgical resection. The tumours were classified according to tumour type, grade and stage. The histological types of all tumours were urothelial carcinoma. Pathological BCa were graded according to the 2004 WHO grading system as low or high grade [16]. Bladder tumors were classified as non-muscle invasive (NMIBC) or muscle-invasive (MIBC). Cancer recurrence and progression were also analyzed, cancer recurrence is the identification of a new tumor in the bladder and progression is defined as, a new tumor with advancement of TNM staging.

MnSOD Ala-9Val polymorphism genotyping

Individuals included in the study had 5 mL of total peripheral blood collected in a sterile tube containing EDTA. Genomic DNA fragments containing the Val16Ala-MnSOD SNP in the human SOD2 gene sequence (GenBank accession no. AY267901) were amplified by PCR. Two primer pairs were used in the same reaction to evaluate the polymorphism according to the tetra-primer ARMS-PCR assay as described by Barbisan et al.: F1 (forward) 5'-

CACCAGCACTAGCAGCATGT–3'; F2 (forward) 5'-
 GCAGGCAGCTGGCTaCGGI–3'; R1 (reverse) 5'-
 ACGCCTCCTGGTACTTCTCC–3'; R2 (reverse) 5'-
 CCTGGAGCCCAGATACCCtAAAG–3' [17]. Underlined lower case bases indicate the introduced mismatches. A 514 bp fragment was always obtained as a control for the success of the amplification using the F1 and R1 primer pair, while F2 and R2 were used to access the Val16Ala-MnSOD SNP based on nucleotide substitution at position 2246 on the SOD2 gene.

The tetra-primer ARMS-PCR was carried out with a Phusion Blood Direct Kit® (Thermo Fischer) which amplifies DNA fragments from 1 µL of the total blood sample without previous DNA isolation. The reaction was conducted in a total volume of 40 µL following the manufacturer's recommendations. The PCR amplification was carried out with an initial denaturation at 94 °C for 7 min, followed by 35 cycles of 60 s of denaturation at 94 °C, 20 s of annealing at 60 °C and 30 s of extension at 72 °C, and an additional 7 min of extension at 72 °C at the end of the final cycle. A 20 µL aliquot of amplicon was mixed with 2 µL of loading buffer and resolved by electrophoresis in a 1.5% agarose gel. This procedure resulted in three bands in heterozygotes (514, 366 and 189 bp) and two bands in homozygotes (Val/Val resulting in bands of 514 and 189 bp, and Ala/Ala resulting in bands of 514 and 366 bp).

Statistical analysis

The data was analyzed with SPSS 18.0 software (Chicago, USA). Student's t-test was performed to compare age between BCa patients and healthy controls. Hardy-Weinberg equilibrium (HWE) was calculated by chi-

square test. To verify the association between Val16Ala-MnSOD SNP and the main BCa prognostic factors (cancer invasion, histological grade, and disease recurrence, smoking status, sex and age) were performed Pearson's chi-squared tests and the interaction effect were measured by stratification. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated using a logistic regression model. Recurrence outcome was analyzed by Kaplan–Meier survival curve. The statistical significance was established as 5% ($P < 0.05$).

Results

Table 1 showed demographic parameters of control and bladder cancer patients. BCa risk was found to be significantly associated with men patients (OR = 1.951, 95% CI = 1.172–3.248; $P = 0.009$).

Table 1 Demographic parameters of controls and bladder cancer patients

Variables	Controls <i>n</i> = 247	Bladder Cancer <i>n</i> = 110	<i>P</i> value
Sex			0.009
Male	154 (62.3%)	84 (76.4%)	
Female	93 (37.7%)	26 (23.6%)	
Age	70.5 ± 7.7	70.2 ± 10.6	0.0776
Grade			
High grade		42 (39.3%)	
Low grade		65 (60.7%)	
TNM			
Tcis		1 (0.9%)	
Ta		27 (24.5%)	
T1		56 (50.9%)	
T2		22 (20.0%)	
T3		4 (3.6%)	
Smoker yes		97 (88.2%)	
Smoker no		13 (11.8%)	
Cancer invasion			
NMIBC		84 (76.4%)	
MIBC		26 (23.6%)	

Demographic parameters of the controls and bladder cancer patients. TNM = cancer staging system, Tcis = carcinoma *in situ*, Ta = non invasive papillary carcinoma, T1 = tumor has spread to subepithelial connective tissue (lamina propria), T2 = the tumor has spread to the muscle of the bladder wall, T3 = tumor has grown into the perivesical tissue, NMIBC = non-muscle-invasive bladder cancer and MIBC = muscle-invasive bladder cancer

The genetic frequencies of the Val16Ala-MnSOD SNP were consistent with the HWE ($P = 0.422$). Allele frequencies in the sample were $A = 0.49$ and $V = 0.51$.

The frequencies of the MnSOD genotypes were presented in Table 2. When we compared alleles, data showed a risk among subjects with the Ala allele compared to Val carriers (OR = 2.31, 95% CI = 1.38–3.85; P = 0.001).

Table 2 MnSOD genotype distribution in controls and bladder cancer patients

Val16Ala-MnSOD SNP	Controls <i>n</i> (%)	Bladder Cancer <i>n</i> (%)	OR (95% CI)	<i>P</i> value
Val/Val	61 (24.7%)	28 (25.5%)		
Ala/Val	141 (57.1%)	45 (40.9%)		
Ala/Ala	45 (18.2%)	37 (33.6%)		
Val/Val + Ala/Val	202 (81.8%)	73 (66.4%)	1.00	
Ala/Ala	45 (18.2%)	37 (33.6%)	2.31 (1.38 – 3.85)	0.001

MnSOD genotype distribution in controls and bladder cancer patients. MnSOD = manganese superoxide dismutase, Ala = alanine, Val = valine, OR = odds ratio, CI = confidence interval.

Invasiveness of the BCa was observed in figure 1. BCa patients with a Val/Val genotype had a tendency for association with NMIBC (P = 0.054).

Figure 1 MnSOD genotype in NMIBC and MIBC

Patients Ala carriers (Ala/Ala and Ala/Val) showed an increase in risk but not statistically significant, of developing invasive BCa compared to those with the Val/Val genotype (OR = 3.25, 95% CI = 0.7–15.15; P = 0.133).

When stratified according to cellular grade of differentiation, no association was observed between each genotype distribution of MnSOD (P = 0.357).

Multivariate analyses were performed according to patients' smoking status and no association related to grade and invasion between Val16Ala-MnSOD SNP genotypes was observed in smokers and non-smokers patients.

Bladder cancer recurrence occurred in 57 patients (51.8%) and posterior progression cases were demonstrated in figure 2.

Figure 2 Recurrence and progression cases of bladder cancer patients

Recurrence cases according to Ala/Ala, Val/Val and Ala/Val patients were 20 (54.1%), 15 (53.6%) and 22 (48.9%), respectively. Presence or absence of recurrence was not related to any of the MnSOD genotypes ($P = 0.877$).

There is no association in the time of the first recurrence among the genotypes studied ($P = 0.916$), data showed in Figure 3. The mean time of the first recurrence was 73.5 ± 8.2 months (CI 95% = 4.8 to 7.5 years) and Val/Val patients had a lower average time from the date of diagnosis to first recurrence (53.5 ± 11.5 months CI 95% = 2.6 to 6.3 years).

Figure 3 Time to the first recurrence in all MnSOD genotypes

Discussion

Genetic susceptibility to cancer has been a research focus in the scientific community. Recently, polymorphic variants of the MnSOD gene have drawn more attention in the etiology of several cancers [18]. Val16Ala-MnSOD SNP has been found to disrupt proper targeting of the enzyme from the cytosol to the mitochondrial matrix, where it acts on $O_2^{\cdot -}$ to dismutate it to H_2O_2 . The change in $O_2^{\cdot -}$ and H_2O_2 levels in mitochondria modulates the molecular mechanisms of apoptosis, cell adhesion and cell proliferation [19]. Thus, this polymorphism was associated with the development of several kinds of cancer: prostate [20,21], breast [22], lung[23], skin[24] and bladder [25].

Although the Ala/Ala genotype seems to have antioxidant benefit, with its high mitochondrial activity, studies have suggested increased cancer risk among Ala/Ala subjects [26,27]. We found an important association between the Ala/Ala genotype and risk of BCa (OD = 2.31) when compared with Val carriers. Dal Berto et al. have done a comparison between MnSOD genotypes, which showed a higher frequency of the Ala allele in prostate cancer subjects when compared to control subjects [21].

In our study, association between Ala-9Val polymorphism and BCa was not observed, either in smokers or non-smokers. Maybe more criteria data from smoking status could have been recorded in our study to correlate this association. Woodson et al. showed that Finnish male heavy smokers who carried the Ala/Ala genotype had a 70% increased prostate cancer risk and three-fold increased risk of high-grade tumours compared with patients who carried the Ala/Val and Val/Val genotypes [28]. Contrary to this finding, the study by Hung et al. found that the MnSOD Val/Val genotype increased the risk of BCa, and this association was restricted to smokers, particularly heavy smokers [25].

A prospectively designed study performed by Kucukgergin et al. stratified data according to the grade of BCa, and no statistically significant difference was observed between the distribution of each MnSOD genotype. BCa patients with the Ala/Ala genotype of MnSOD did not have an increased risk of high-grade tumours compared to patients with the Val/Val genotype [29]. Our case-control study did not find association between BCa grade and genotype distribution of MnSOD. However, we found a tendency association between Val/Val genotype and the occurrence of NMIBC. Associations between high cancer stage and

MnSOD genotype are in constant conflict. A previous study reported that the Ala/Ala genotype was a predictive factor for developing advanced prostate cancer [15] but another recent case-control analysis disagreed, showing an association with Val-allele carriers and aggressive prostate cancer at the time of diagnosis [21].

An important analysis performed by our group was the recurrence monitoring of the BCa patients. Although we did not find association between presence or absence of recurrence and difference in time of the first recurrence and MnSOD genotype, these analyses are the key area for research in BCa, taking into consideration the fact that it is a heterogeneous neoplasia that presents high probability of recurrence and progression and low rates of metastasis and mortality. In other cancers, such as breast cancer association between aggressiveness and survival was observed in relation to Val16Ala-SOD2 SNP. However, whereas AA increased the risk to develop breast cancer, VV increased the risk of metastasis and mortality [20, 30]

Finally, it is important to comment some methodological constraints related with this study, mainly in respect to patients sample number that is not so large. However, it is reflex of the incidence of BCa patients during the period of study. In these terms, it was not possible to affirm that no exist association between Val16Ala-MnSOD SNP and some variables, such as cancer aggressiveness is consequence of reduced number of patients. Therefore, complementary investigations we higher number of BCa patients need to be performed to clarify this question. Despite this limitation, we believe that results described here are epidemiologically relevant to be reported.

Conclusion

ROS may play an important role in bladder carcinogenesis. Val16Ala-MnSOD2 SNP, which causes a cellular superoxide-hydrogen peroxide imbalance, could modulate individual susceptibility to BCa. The high MnSOD efficient genotype (Ala/Ala) could be considered as a risk factor for BCa. Exogenous exposure that may impact oxidative burden does not seem to modify the effects of these genotypes in this population. Additional studies with a larger number of subjects and more detailed data from smoking status and dietary habits are needed to clarify antioxidant gene–gene interactions and antioxidant gene environmental risk factors in BCa.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ADR, TMG, CGB, IBMC designed research. ADR, JNLS, YMTS, MMA, MMMF performed research. ADR, CGB and IBMC analyzed data. ADR, YMTS, MMA wrote paper. All authors read and approved the final manuscript.

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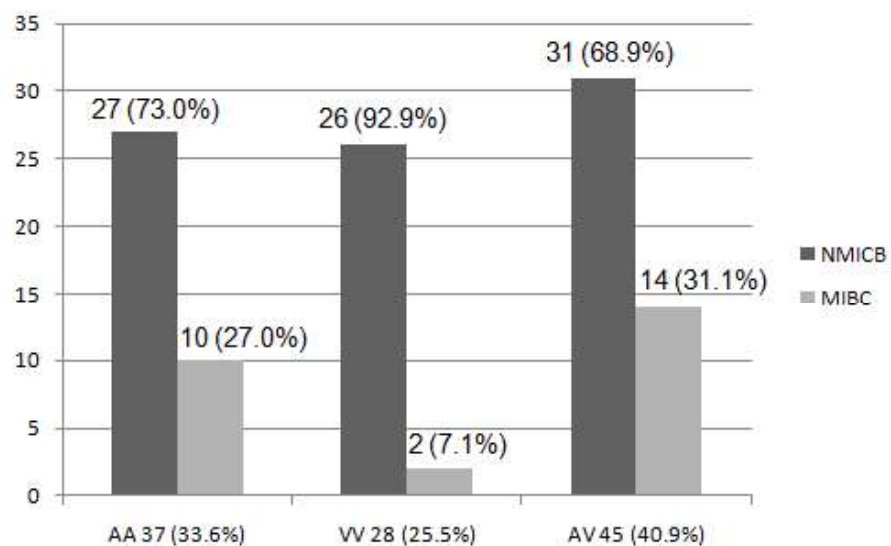
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Figure title and legend section:

Figure 1 MnSOD genotype in NMIBC and MIBC. Legend: MnSOD genotype in NMIBC and MIBC. NMICB = non-muscle-invasive bladder cancer, MIBC = muscle-invasive bladder cancer, AA = alanine/alanine, VV = valine/valine, AV = alanine/valine.

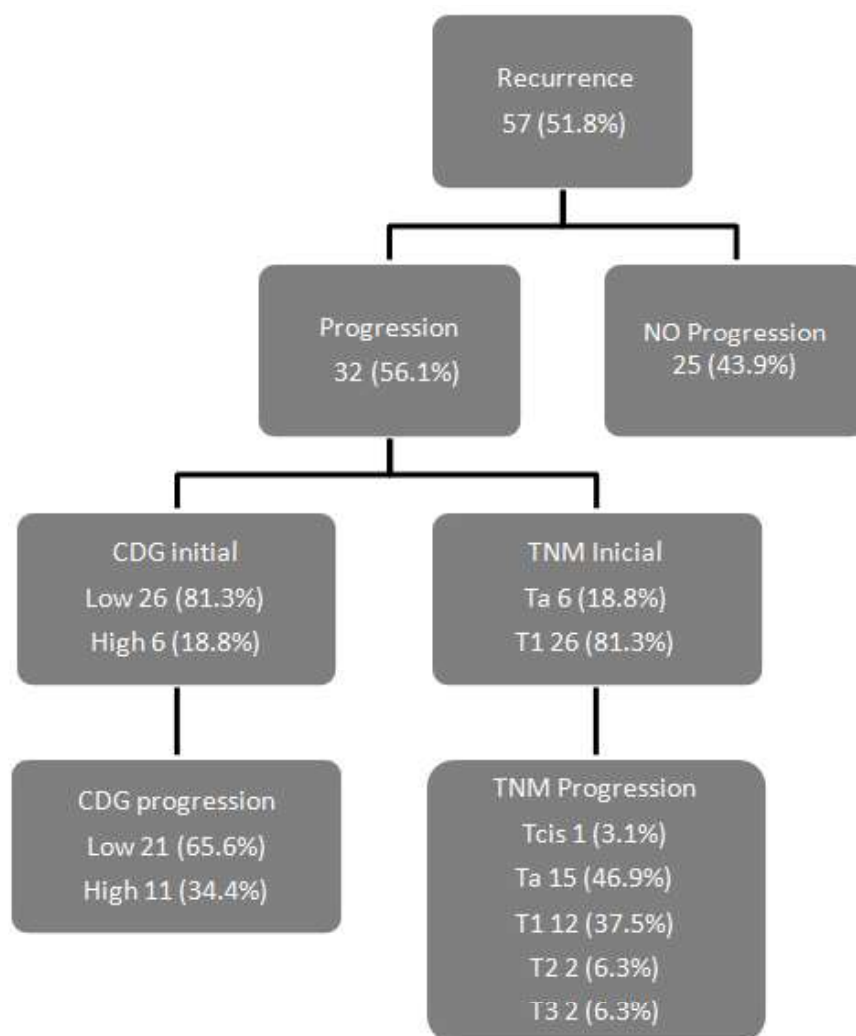
Figure 2 Recurrence and progression cases of bladder cancer patients. Legend: Recurrence and progression cases of bladder cancer patients. CDG = cellular differentiation grade, TNM = cancer staging system, Tcis = carcinoma in situ, Ta = non invasive papillary carcinoma, T1 = tumor has spread to subepithelial connective tissue (lamina propria), T2 = the tumor has spread to the muscle of the bladder wall, T3 = tumor has grown into the perivesical tissue.

Figure 3 Time to the first recurrence in all MnSOD genotypes. Legend: Cum Rec = cumulative recurrence, SOD = superoxide dismutase, AA = alanine/alanine, VV = valine/valine, AV = alanine/valine.

Figure 1

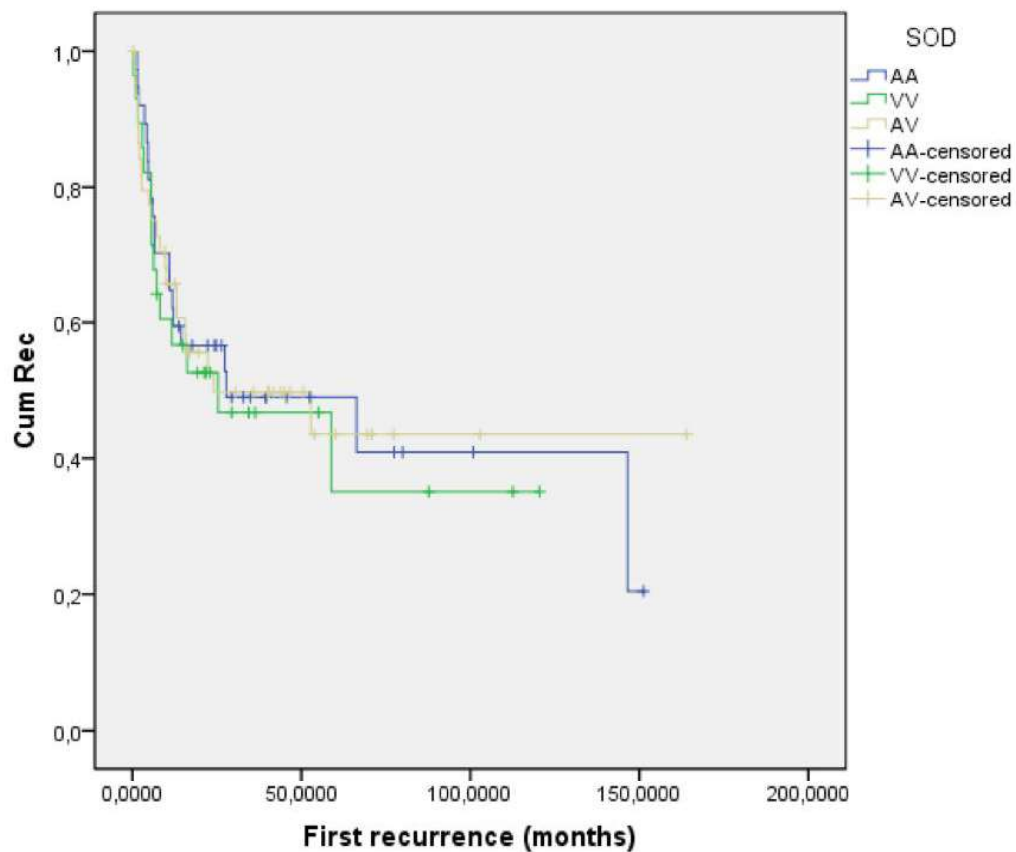
MnSOD genotype in NMIBC and MIBC. NMIBC = non-muscle-invasive bladder cancer, MIBC = muscle-invasive bladder cancer, AA = alanine/alanine, VV = valine/valine, AV = alanine/valine.

Figure 2



Recurrence and progression cases of bladder cancer patients. CDG = cellular differentiation grade, TNM = cancer staging system, Tcis = carcinoma *in situ*, Ta = non invasive papillary carcinoma, T1 = tumor has spread to subepithelial connective tissue (lamina propria), T2 = the tumor has spread to the muscle of the bladder wall, T3 = tumor has grown into the perivesical tissue.

Figure 3



Time to the first recurrence in all MnSOD genotypes. Cum Rec = cumulative recurrence, SOD = superoxide dismutase, AA = alanine/alanine, VV = valine/valine, AV = alanine/valine.

4 CONSIDERAÇÕES FINAIS

4.1 CONSIDERAÇÕES FINAIS DA TESE

Os trabalhos aqui apresentados fazem parte de um grande projeto intitulado “**Estudo de biomarcadores e do polimorfismo da MnSOD em pacientes com câncer de bexiga**”, aprovado pelos Comitês de Ética em Pesquisa (CEP) da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), com Parecer N° 258.491 e ISCMPA com parecer N° 226.953. Deste projeto maior foram desenvolvidos trabalhos na modalidade de Iniciação Científica, uma monografia de Graduação, a presente Tese de Doutorado, além de outros projetos em andamento.

O Serviço de Urologia do Hospital Santa Rita (HSR) da ISCMPA é um centro de referência no tratamento de pacientes com câncer de bexiga, assim como o serviço de Patologia, na excelência do diagnóstico. Esta parceria, para o desenvolvimento deste projeto teve início em 2010, no serviço de Patologia, e em 2013 no Serviço de Urologia, sendo um objetivo comum a ambos os grupos, além do diagnóstico e tratamento, a investigação de fatores de risco e biomarcadores desta neoplasia que onera tanto o sistema de saúde.

O vínculo entre uma instituição de ensino (UFCSPA), e um hospital referência (HSR – ISCMPA) é extremamente promissor e deve ser incentivado, pois diversos trabalhos científicos podem emergir, gerando publicações e principalmente levando à informação a população.

Outro ponto positivo a ser considerado é que durante o desenvolvimento do projeto contamos com o auxílio de alunos da Biomedicina e Medicina, possibilitando a interação entre a graduação e a pós-graduação a partir da Iniciação Científica (IC). Como resultado desta profícua relação, participamos de

dois eventos da área ambos premiados como: 2º lugar no XII Congresso Sul Brasileiro de Urologia e trabalho destaque na categoria “Trabalhos relacionados à pesquisa” durante a “I Mostra de trabalhos de ensino, pesquisa e extensão da UFCSPA.”

Apresentamos como resultados desta pesquisa, o Artigo I, intitulado “Immunohistochemistry Biomarkers in Nonmuscle Invasive Bladder Cancer” no qual revisamos os marcadores imunoistoquímicos recentes utilizados no diagnóstico e prognóstico do NMICB. O artigo foi publicado na revista *Applied Immunohistochemistry and Molecular Morphology* em novembro de 2015.

O Artigo II, intitulado “Cell cycle markers in the evaluation of bladder cancer”, concluímos que as proteínas regulatórias da proliferação e ciclo celular são potenciais biomarcadores prognósticos para uso clínico-patológico no câncer de bexiga e um achado importante mostrou que a expressão alterada de mais de um marcador de ciclo e proliferação celular podem determinar a recorrência do câncer de bexiga. O artigo científico foi submetido para a revista *Pathology and Oncology Research*.

O Artigo III, intitulado “Association between bladder cancer risk and genetic variants of MnSOD enzyme”, revela como principal achado que o genótipo Ala/Ala da MnSOD pode ser considerado como fator de risco para o câncer de bexiga. O artigo científico foi submetido para a revista *Journal of Biomedical Science*.

O Ca de bexiga é uma neoplasia heterogênea que apresenta alta probabilidade de recorrência e progressão (apenas 15% evoluem para metástase e invasão). Assim, é de extrema importância que a sua identificação

e estratificação clínico patológica seja feita com a maior precisão possível, permitindo uma modulação adequada da doença. Biomarcadores atuam neste contexto para aumentar a precisão do prognóstico e consequentemente permitindo um tratamento individualizado mais eficaz. No entanto, as anormalidades moleculares do Ca de bexiga são altamente complexas, sendo assim pouco provável que um único marcador seja capaz de segregar com precisão tumores de fenótipos clínico patológicos semelhantes em categorias prognósticas precisas. Como demonstrado pelos nossos dois estudos, de revisão e de coorte, a combinação de biomarcadores independentes, porém complementares, pode permitir um diagnóstico ou prognóstico mais preciso que um marcador isolado, sendo um tópico importante para investigação a pesquisa por um painel de biomarcadores IHQ. Em adição, sabe-se que EROs podem desempenhar um papel importante na carcinogênese da bexiga e a susceptibilidade individual ao câncer pode ser modulada pelo polimorfismo da MnSOD, sendo assim, demonstramos que o genótipo Ala/Ala da MnSOD pode ser considerado como um fator de risco para o câncer da bexiga. Novos estudos devem ser conduzidos correlacionando estresse oxidativo, fatores ambientais e genéticos ao risco de recorrência, progressão e desenvolvimento do Ca de bexiga.

No **Apêndice 6.1** desta Tese, apresentamos o artigo intitulado “Correlation between the presence of degenerated inclusion-bearing cells in voided urine samples and occurrence of polyomavirus infection”. Esse projeto foi realizado em parceria com o Prof. Dr. Alessandro Pasqualoto e Dr João Carlos Prolla. Anteriormente a este projeto, a dissertação de mestrado publicada pela aluna Alana Ranzi tinha como assunto a pesquisa de células “decoy” em

pacientes transplantados renais, havendo assim uma relação com o presente artigo e necessidade da continuação de estudos na área de transplante renal e citopatologia. O artigo foi aceito recentemente pela revista Cytopathology.

No **Anexo 7.1**, incorporamos o artigo intitulado “Immunosuppression and the occurrence of HPV in kidney transplant patients verified by urinary cytology”. O presente artigo foi publicado no Jornal Brasileiro de Patologia e Medicina Laboratorial em fevereiro de 2016. Este projeto foi desenvolvido na época pela então aluna de Biomedicina Rogéria M. Leon, orientado pela Prof. Dra. Claudia Bica e co-orientado pela aluna de doutorado Alana Ranzi.

Com a participação em congressos e jornadas acadêmicas podemos apresentar resultados parciais deste trabalho de doutorado, ação fundamental na construção do conhecimento.

4.2 CONSIDERAÇÕES FINAIS DO DOUTORAMENTO

A oportunidade de cursar Doutorado nesta Instituição, circundada por uma equipe de professores competentes e atuantes nos três pilares que estruturam a Universidade: pesquisa-ensino-internacionalização permitiu-nos desenvolver diversos trabalhos, os quais abaixo descrevemos:

4.2.1 No âmbito da pesquisa, através dos artigos, apêndices e anexos, publicamos 2 artigos científicos e outros 3 já foram submetidos a revistas internacionais. Além disso, ainda estamos engajados nos seguintes projetos:

- “Avaliação do DNA livre no sangue de pacientes com câncer de bexiga”, em andamento sob responsabilidade do aluno de IC Yuri Strey.

- “Potential urinary diagnostic biomarker EN2 on three large independent cohorts of bladder cancer cases”. Em andamento na University of Surrey sob orientação da Dra Nicola Annels.

Durante o doutorado o projeto “Estresse oxidativo e o estudo de biomarcadores no câncer de bexiga” foi contemplado com três bolsas de Iniciação Científica, nos anos de 2013, 2014 e 2015. Além disso, co-orientamos alunos de Graduação em seus Trabalhos de Conclusão de Curso, intitulados:

- Expressão imunoistoquímica das proteínas p16, p21, p27, p53, pRb e Ki-67 em pacientes com carcinoma urotelial de bexiga, (novembro de 2014);
- Fatores de risco associados à prevalência de HPV em pacientes transplantados renais a partir de exame citológico urinário, (novembro de 2014).

Fomos convidadas a contribuir no Livro Cinema, Ética e Saúde, pelos organizadores: Ana Carolina da Costa Fonseca, Cora Efrom, Isabella Moreira dos Santos, publicado pela Editora Bestiário (2014). Neste livro, o seguinte capítulo foi escrito: *AIDS, preconceito e tratamento em “Clube de Compras Dallas”*. Da autoria de Alana Ranzi, Giovana Tavares dos Santos, Rosicler L. Brackmann e Claudia G. Bica.

4.2.2 No âmbito do ensino, em de agosto de 2015, recebi o convite para ministrar aulas de Pesquisa Clínica para o curso de Pós Graduação em Pesquisa Clínica do Hospital Moinhos de Vento em Porto Alegre, sendo que em setembro de 2015 fui professora responsável (preceptora) pela condução do estágio em pesquisa clínica da aluna Munique Azevedo no HNSC. Desta forma, mostramos o diferencial que este curso de Pós-Graduação oferece em nossa formação profissional e inserção no mercado de trabalho.

Na própria Instituição (UFCSPA) como membro da comissão organizadora, auxiliamos na promoção do evento: IX Jornadas do Programa de Pós- Graduação em Patologia, na edição de 2013.

No ano de 2015 participei da X Jornada do Programa de Pós Graduação em Patologia, na qualidade de palestrante do tema: Experiências exitosas de pesquisadores brasileiros no exterior.

4.2.3 No âmbito da internacionalização, em agosto de 2014 através do Programa Institucional de Bolsas de Doutorado Sanduíche no Exterior (PDSE), realizei meu intercâmbio de seis meses na University of Surrey/ Inglaterra, com o projeto intitulado: “Pesquisa de novos marcadores tumorais para o câncer de bexiga”. Os projetos desenvolvidos estão apresentados no **anexo 7.2** desta tese e como resultado desta importante parceria um artigo científico analisando três distintas coortes de pacientes com câncer de bexiga será publicado em abril/2016.

Acredito que o PDSE além de oportunizar alunos a grandiosa experiência do intercâmbio, cria vínculos entre instituições de alto padrão científico e de ensino, como a Universidade de Surrey e a UFCSPA. O intercâmbio na vida de um acadêmico, além do desenvolvimento de habilidades técnicas, aprimoramento da língua estrangeira e crescimento pessoal, é uma alternativa conveniente de estabelecer contatos e emergir profissionalmente. Com um trabalho bem realizado, dedicação integral e interesse do aluno o futuro se torna promissor tanto no país de origem quanto fora do país.

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6 APÊNDICES

6.1 Artigo aceito pela revista *Cytopathology*: “Correlation between the presence of degenerated inclusion bearing cells in voided urine samples and occurrence of polyomavirus infection.”

Cytopathology

Correlation between the presence of degenerated inclusion-bearing cells in voided urine samples and occurrence of polyomavirus infection.

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Key Words:	polyomavirus, kidney transplantation, voided urine, degenerated inclusion-bearing cells

Title page

Correlation between the presence of degenerated inclusion-bearing cells
in voided urine samples and occurrence of polyomavirus infection.

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Abstract

Objective: The purpose of the present prospective cohort study was to monitor urine cytology samples from recipients of renal transplants in order to search for the occurrence of decoy cells and degenerated inclusion-bearing cells with an aim to correlate the existence of these cells with molecular detection of BKV DNA in urine.

Material and Methods: This study included patients who underwent renal transplantation. Patients had their urine tested quarterly, during the first year post-transplantation, for the presence of decoy cells and degenerated cells, as well as by quantitative determination of BKV load in the urine and plasma. Results: 361 examinations were performed on 101 patients within 12 months of attendance. Urine cytology results were: 198 (54.9%) negative and 60 (16.6%) positive for the presence of viral cytopathic effects depending on the presence of BKV infection, 72 (19.9%) positive for the manifestation of degenerated cells, and 31 (8.6%) unsatisfactory for analysis. There was a subtle tendency toward the presence of degenerated inclusion-bearing cells in cases in which the virus was detected in voided urine. However, the presence of degenerated cells exhibited an inclination to BKV positivity in months 3, 6 and 9; and exclusively in month 12, this trend was statistically significant. Conclusions: There were not enough strong morphological and staining elements to state the origin of the degenerated cells or to describe the nature of the infection (viral or bacterial), given that these cells were undergoing an apoptotic process in post renal transplant patients.

Introduction

BK virus (BKV) is a polyomavirus that belongs to the Polyomaviridae family, which also includes JC virus (JCV) and Simian Virus 40 (SV40) (1). The BKV is the causative agent of polyomavirus-associated nephropathy, which occurs when the virus reactivates from a latent state due to immunosuppressive therapy after renal transplantation (2, 3, 4).

The diagnosis of nephropathy due to BKV (BKVN) is made by renal biopsy, although other assays, such as urine cytology (decoy cells), immunohistochemistry and quantification of the viral load in blood and urine by polymerase chain reaction (PCR), have been used for screening of BKV replication. These assays, used in combination with biopsy, enhance the diagnosis of BKVN (5, 6).

According to Singh *et al.* and Ariyasu *et al.*, “decoy cells” is a name used for epithelial cells with intranuclear viral inclusions that can have distinct phenotypes (types 1-4) depending on the state of viral replication and maturation as well as the specific traits of the cell architecture. There are four different morphologic categories of decoy cells (7, 8). The classical decoy cell belonging to type 1 shows homogenous, amorphous and hazy intranuclear inclusion bodies. The nucleus usually has an eccentric position in relation to the cytoplasm. The type 2, so-called CMV-like, decoy cell shows central, intranuclear viral inclusion bodies involving by irregular halos. The nuclear periphery is clearly recognisable. This morphotype was not registered during the present study. The type 3 decoy cell is multinucleated and exhibits chromatin with a granular aspect. Type 4 has a vesicular nucleus, where it is possible to observe clumps interspaced by granular chromatin.

The sequence in which these phenotypes may happen in the course of intranuclear viral assembly is unknown (7). The origin of decoy cells cannot be clearly

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3 established by considering only their morphologic features. It seems reasonable that
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5 they would often originate from the urothelium in healthy and asymptomatic cases.
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7 However, in patients with BKN, decoy cells probably come from the renal parenchyma,
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9 leading to a speculative hypothesis that proposes that BKVN may be a consequence of
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11 an ascending route of infection with dispersion of polyomavirus replication from
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13 transitional cell layers of the bladder to the tubular epithelium of the kidney in certain
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15 patients (7).
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19 Urine cytology allows the early identification of BKV infection, providing the
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21 opportunity for an intervention involving the reduction of immunosuppression in order
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23 to control and prevent the replication of BKV and thus reduce the risk of organ rejection
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25 (9). Microscopic examination of the urine, using Papanicolaou staining to identify the
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27 decoy cells, could also find degenerated inclusion-bearing cells (degenerated cells) in
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29 renal transplant patients. These cells were studied by our research group, as the
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31 literature does not report the appearance of them and there is no existence of a clinical
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33 pathological correlation with the infection by polyomavirus or loss of the graft.
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37 The purpose of the present prospective study was to monitor urine cytology for
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39 the presence of decoy cells and degenerated cells in renal transplant recipients and to
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41 correlate the findings of these cells with molecular detection of viral DNA in urine.
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45 **Material and methods**

46 **Patients**

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49 This study included male and female patients (≥ 18 years) who underwent renal
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51 transplantation at Irmandade da Santa Casa de Misericórdia de Porto Alegre (ISCMPA).
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53 The patients who were included in this study (from June 2012) had their urine tested
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55 quarterly during the first year post-transplantation for the presence of decoy cells and
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3 degenerated cells as well as for quantitative determination of BKV load in the urine
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5 (viruria). All patients with evidence of urinary BKV replication (decoy cells and/or
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7 DNA BKV in urine) were subjected to qPCR (Real-Time PCR) for BKV in plasma
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9 samples (viremia). Patients' charts were reviewed to collect demographic and clinical
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11 data.
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15 We included all renal transplant recipients who consented to participate in the
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17 study. The study was approved by the Institutional Review Board (protocol numbers
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19 3531/11 and 915/12) and followed the guidelines and regulatory standards for research
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21 involving human subjects of the Brazilian National Health Council.
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25 Urinary Cytopathology Protocol
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28 The urine sample collected was the midstream of the first voided urine in the
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30 morning. 10 ml of urine was centrifuged at 1.300 RPM for 10 min. The supernatant was
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32 removed, and the pellet was cytocentrifuged (cytospin) at 800 RPM for 6 min. Two
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34 smears were subjected to Papanicolaou staining. The whole circular area of the cytospin
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36 (5 mm diameter) was screened with a microscopic enlargement of 200x for the presence
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38 of decoy cells and degenerated cells. All urine cytology examinations were evaluated by
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40 the same cytopathologist at the Laboratory of Pathology – ISCMPA. Cells with a large
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42 nucleus totally occupied by a viral inclusion body or homogeneous ground-glass
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44 chromatin, with or without a cytoplasmic halo, were classified as decoy cells. Urine
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46 cytology results were organised into four groups: positive (identified as decoy cells),
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48 degenerated cells (identified as degenerated inclusion-bearing cells), unsatisfactory for
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50 analysis (findings with an excess of white blood cells, erythrocytes or crystals) and
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52 negative (none of the above).
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qPCR test

Urine samples were centrifuged, and DNA was extracted using QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The methodology used for qPCR testing was described in a previous communication (10).

Results

Sample of the study

The study included 101 adult patients (older than 18 years of age) who had undergone renal transplant by a transplant specialist in a hospital in southern Brazil. After signing an informed consent form, the patients were followed for a period of 12 months (months 3, 6, 9, 12) post renal transplant.

Of 101 patients, 56 (55.4%) were men and 45 (44.6%) were women, and the average age was 46.9 years, with a minimum age of 19 years and maximum of 76. Regarding the status of the patients, 82 (81.2%) remained in attendance, 11 (10.9%) failed to attend, 4 (4%) lost the graft and 4 (4%) died.

Urinary cytology examination

Three hundred and sixty one examinations were done, originating from 101 patients during a 12 month period. Of these 198 (54.9%) turned out to be negative, 60 (16.6%) showed the presence of viral cytopathic effects of the type polyomavirus, 72 (19.9%) had the presence of degenerated cells and 31 (8.6%) were unsatisfactory for analysis.

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qPCR

The analysis of BKV by qPCR was carried out in 362 samples of urine, of which 112 (31%) were negative, 99 (27%) were positive and 151 (42%) had results ≥ 154 copies of viral DNA. All positive viremia patients were positive for viruria, but not all viruria was positive for viremia (Table 1).

Nephropathy due to BKV

BKVN was diagnosed using the gold standard test (biopsy) in three (2.97%) patients, all of whom presented positive decoy cells, positive viremia, positive viruria and ≥ 154 copies of viral DNA. One patient presented with interstitial nephritis through cytomegalovirus associated with rare viral inclusions by BKV in month 3, losing the organ soon after the third month of attendance.

Degenerated inclusion-bearing cells

The inclusion-bearing cells exhibited degenerative changes in the cytoplasm, which appeared vacuolated and slightly foamy, suggesting an apoptotic scenario. It is important to note that the cytoplasmic inclusion bodies were frequently stained red or blue by the Papanicolaou technique (Figure 1).

The results found and presented in the Figures 2, 3, 4 and 5 show the tendency of the degenerated cells and the virus positivity in the urine (according to confirmation by qPCR). Although the presence of degenerated cells indicate a positive tendency for BKV in months 3, 6 and 9, they were only statistically significant in month 12.

Discussion

BKV infection continues to be a significant reason for allograft dysfunction in renal transplant recipients. Prince *et al.*, in 2009, reported that the prevalence of

polyomavirus nephropathy was 3.3%. In our study, 3 (2.97%) patients were diagnosed with BKVN using the gold standard test renal biopsy (11).

Even if renal biopsy has been considered the gold standard for the diagnosis of BKV nephropathy, the use of noninvasive techniques, such as the search for decoy cells and the qualitative and quantitative determinations of BKV DNA in serum and urine, might help in selecting patients at greater risk of suffering from a nephropathy, which would lead to loss of the organ (12). But these methods cannot replace biopsy (13).

Using the qPCR technique, it was noticed that the average proportion of patients who presented viruria was 68.2% during attendance, but this was gradually reduced after the sixth month of attendance. That may be due to modulation of the immunosuppression carried out by the medical team during the observed period. The average viremia noticed at months 3, 6, 9 and 12 was 15.7%, with this figure both increasing and diminishing over the year. With similar finds for viremia, Hirsch *et al.* observed that BKV viremia detected by PCR and BKVN detected by histopathology were found in 13%, and 8% of renal transplant recipients, respectively, and noted that BKV viremia appeared several weeks to months prior to the histopathological changes of BKVN. They concluded that monitoring of viruria and viremia by PCR was useful in identifying patients at risk for BKVN since immunosuppressive therapy for such patients could be tailored for those with viremia (14).

In keeping with our findings of viruria, Montagner *et al.* observed among 120 renal recipients under graft dysfunction, the prevalence of viruria was 61.7% (95% CI: 52.4-70.4), suggesting that an intense viruria predicts a viremia and probably a nephropathy by virus (15). In our study, 3 patients with nephropathy by polyomavirus had a viral replication viruria with ≥ 154 copies of viral DNA. Our study observed that

all patients with viremia showed positive viruria, corroborating with the literature that 100% of transplant recipients with tubular interstitial nephropathy and positive viremia always have a positive urinary PCR result (13, 16).

In renal transplant recipients, the prevalence of decoy cells varies from 9% to 18% (17, 18, 19, 20). In the present report, 16.6% of graft recipients had a positive presence of BKV, most of the patients had urinary cytology negative for BKV (54.9 %) and 19.9% of patients showed the presence of degenerated cells. A study carried out by our group in 2012 evaluated 1,713 urinary cytopathologic examinations of renal-transplanted patients, and the results of the examinations were 45.8% negative, 24.9% positive and 22.2% presence of degenerated cells (9). Comparing with the study of Koukoulaki *et al.*, all patients with viral load in urine had the presence of decoy cells, but all decoy cells in urine did not show viruria confirmed by PCR, raising the possibility that the decoy cells had undergone apoptosis and lost their viral DNA. Urine cytology is expected to be less sensitive when compared to molecular methods in identifying viral DNA (21).

It is hard to differentiate between renal tubular epithelial cells and urothelial cells when investigating exclusively cytological structures without using specific antibodies to distinguish these cells from each other (8). So it would be arbitrary to state precisely the origin of the degenerated inclusion-bearing cells reported here. In addition, the cytoplasmic bodies of the degenerated cells displayed moderate to excessive red pigmentation. Unfortunately, the recognition of nuclear and/or cytoplasmic inclusions, in cells undergoing an apoptotic process, does not provide a specific indication of viral infection. Routine cytopathology investigations can reveal that bacteria may also be stained red or blue by the Papanicolaou method (22). It would not be surprising to find the existence of bacterial infections in the presence of an immuno-modulating virus like

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3 BK polyomavirus. Therefore, we do not have enough definitive evidence to state the
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5 origin of the degenerated cells nor to describe the nature of its infection (viral or
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7 bacterial).
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10 **Conclusion**

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13 From our findings it is clear that urine positive for decoy cells requires further
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15 screening with quantitative tests (urine or plasma BK viral load) in order to confirm the
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17 presence of BKV. One cannot assume the presence of BKV-associated nephropathy
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19 based on decoy cells alone. In relation to our findings regarding degenerated cells, in
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21 spite of the tendency of decoy cells to be present in patients with polyomavirus
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23 infection, it doesn't have any clinical value which has been proven statistically. There
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25 were not enough strong morphological and staining elements to state the origin of
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27 degenerated cells or to describe the nature of their infection (viral or bacterial),
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29 considering that these cells were under an apoptotic process in post renal transplant
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31 patients.
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35 **Acknowledgments**

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37 The authors thank Gabriel G. Pinto and José Antonio Tesser Poloni for
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39 performing qPCR tests and patients' recruitment.
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Table 1 (Positivity of BKV by urine cytology and qPCR analyses)

	Month 3	Month 6	Month 9	Month 12
Decoy cells	19.8%	12.9%	14.9%	11.9%
Viruria	74.25%	78.9%	69.7%	50%.
Viremia	18.6%	17.6%	10.8%	15.7%

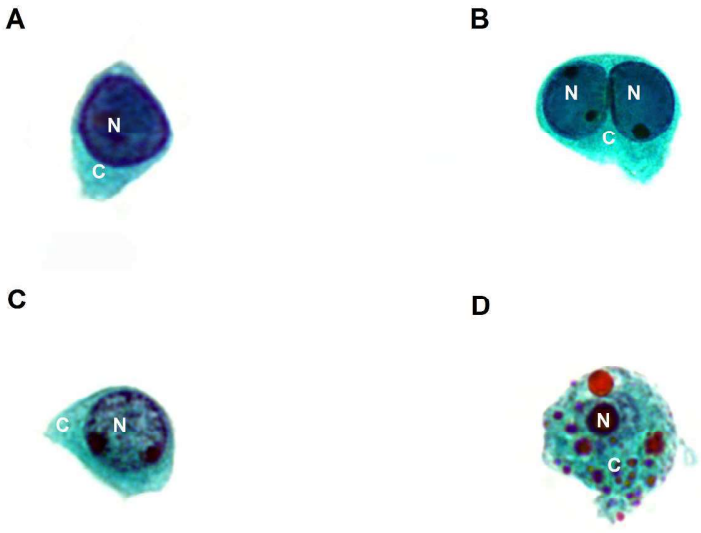


Figure Captions (1): Three categories of decoy cells (a-c) and degenerated inclusion-bearing cells with Papanicolaou staining (d). (a) Type 1, classic decoy cell. (b) Type 3, cell with more than one nucleus. (c) Type 4, cell with clumps of chromatin and an evident nucleolus. (d) Inclusion-bearing cell with degenerative changes in the cytoplasm, which appears vacuolated and slightly foamy suggesting an apoptotic scenario. Magnification: 400X. 1346x965mm (72 x 72 DPI)

Figures (2, 3, 4 e 5) Degenerated Cells X PCR

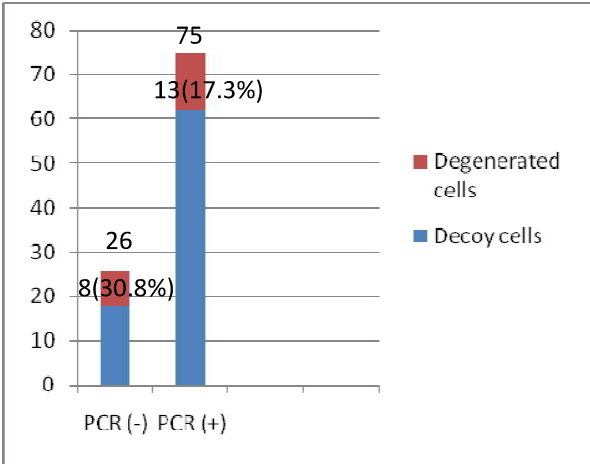


Figure 2 – Month 3 post renal Tx (p = 0,240)

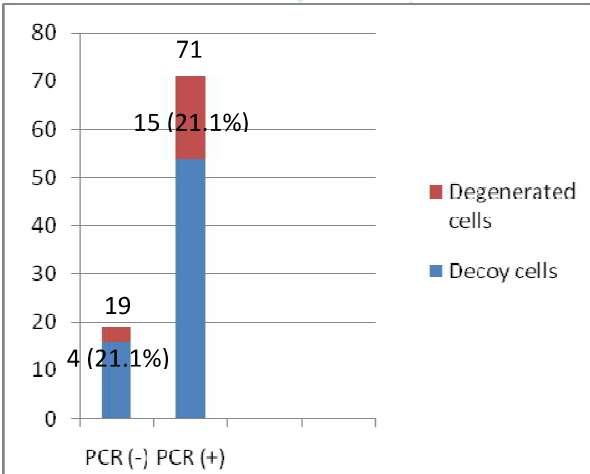


Figure 3 – Month 6 post renal Tx (p=0,382)

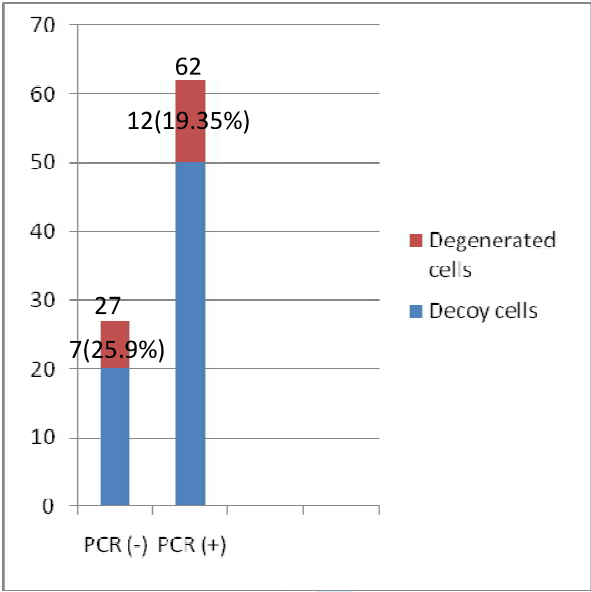


Figure 4 –Month 9 post renal Tx (p = 0,679)

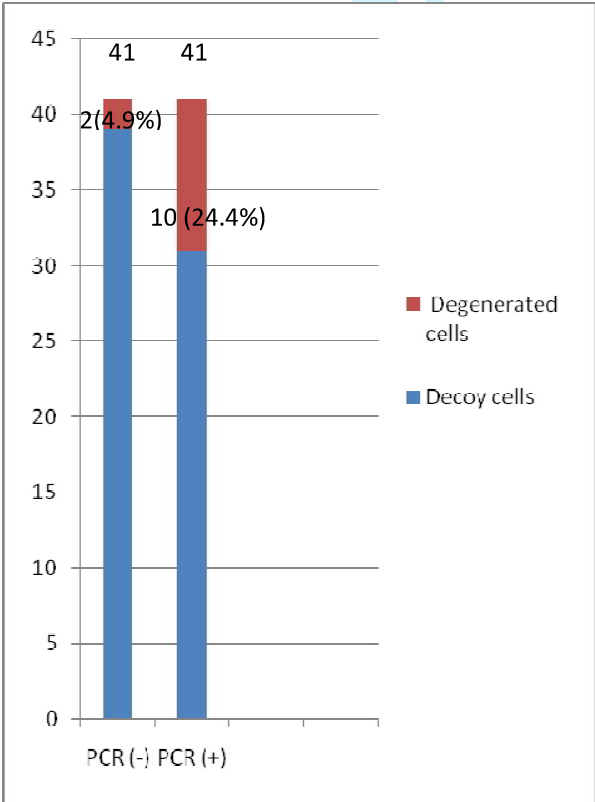


Figure 5 – Month 12 post renal Tx (p= 0,040)

7 ANEXOS

7.1 Artigo publicado: “Immunosuppression and the occurrence of HPV in kidney transplant patients verified by urinary cytology”.

Immunosuppression and the occurrence of HPV in kidney transplant patients verified by urinary cytology

Imunossupressão e ocorrência de HPV em pacientes transplantados renais a partir de exame citológico urinário

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ABSTRACT

Introduction: Human papillomavirus (HPV) is the main cause of cervical cancer, and immunosuppression is recognized as a risk factor for HPV infection and its persistence. After renal transplantation, immunosuppressive agents are used to prevent rejection, but predispose recipients to chronic infections and malignancies. **Objective:** This study aimed to verify, based on urinary cytology (UC), the prevalence of HPV in immunosuppressed kidney transplant patients. **Material and method:** In this cross-sectional study, the population was composed of kidney transplant patients that had undergone routine UC from August 2012 to August 2014. **Results:** There were 2,305 urine cytopathological tests. Thirteen patients with presence of koilocytes in such examination were observed. Therefore, the relative frequency of patients with HPV detected in urine was 0.56%. In the interval until the first post-transplant year, 10 (76.92%) patients presented koilocytes ($p < 0.0001$) in the UC. The dosages of immunosuppressive agents until the first post-transplant consultation, which showed correlation with the period between transplantation and the first UC test with the presence of koilocytes ($p < 0.0001$), were prednisone 10.5-20 mg/day, mycophenolate sodium 901-1,440 mg/day, and tacrolimus 4.5-12 mg/day. **Conclusion:** This study showed immunosuppression as an important risk factor for infection by HPV or its reactivation. Screening UC tests after transplantation may evidence HPV infection.

Key words: immunosuppression; risk factors; kidney transplantation; papillomavirus infections.

INTRODUCTION

Human papillomavirus (HPV) is the main cause of cervical cancer among women, and immunosuppression is recognized as one of the risk factors for HPV infection and its persistence⁽¹⁾. According to Meeuwis *et al.* (2011), HPV prevalence is higher in immunosuppressed women in comparison to their immunocompetent homologues. Immunosuppression probably causes greater susceptibility to HPV infection and/or higher risk of HIV infection persistence⁽²⁾.

Kidney transplant recipients have high risk of developing pre-malignant neoplasms associated with the virus, such as HPV-related anogenital lesions. Most kidney transplant women are known to be infected by HPV, with a 14-fold higher risk of developing cervical cancer, 50-fold higher risk for vulvar cancer, and up to 100-fold higher risk for anal cancer⁽³⁾.

Recognizing HPV infection, by the cell alteration (koilocytosis) observed when this virus is present, became important due to its identification as carcinogenic in the development of squamous cell carcinoma of the genital tract⁽⁴⁾. During the viral course of HPV, viral particles invade the cell nucleus and cause characteristic degenerative alterations⁽⁴⁾. Koilocytosis is considered a pathognomonic sign of HPV infection. The typical koilocytosis presents nuclear enlargement, with reactive changes, and characteristic sharply demarcated halo separated from cytoplasm by a clearly condensed rim⁽⁴⁾.

As reported by Reis *et al.* (2010), HPV infection is frequently common among young adults of both sexes, with estimated prevalence of 20%-46%. HPV dissemination is generally universal among sexually active individuals, with men playing an important role in spreading this virus among women⁽⁵⁾.

Infection by specific HPV types is the main risk factor for cervical cancer. Other risk factors are: early age of first sexual intercourse,

lifetime number of sex partners, promiscuous partners, poor nutrition, parity or multiparity, tobacco smoking, oral contraceptive use, and low socioeconomic status⁽⁵⁻⁸⁾. The host's immune status is another risk factor, as the evolution of cervical lesions may be associated with immune responsiveness. HPV-infected cells lack efficient response to antigens, making their multiplication easy due to delayed recognition by the immune system⁽⁸⁾.

After kidney transplantation, patients must be treated with immunosuppressants to prevent rejection. These drugs have different mechanisms of action, disrupting interaction and/or stimulation of antigen-presenting cells or T-lymphocytes of the human immune system⁽⁹⁾. Immunosuppressive therapy protects the transplanted organ, but predisposes the recipient to chronic infections and malignant diseases. Transplant patients are in risk of cervical intraepithelial neoplasia (CIN) and cervical cancer, due to a decreased immune response, in the case of primary infection, or reactivation of a latent infection, such as HPV of high oncogenic potential⁽¹⁰⁾.

Urine cytology (UC) is an excellent screening method for monitoring kidney transplant patients: it is very convenient, useful and sensitive^(11,12). In accordance with Almeida *et al.* (2006), serial analysis of UC is an adequate screening method⁽¹³⁾.

The present work was aimed at, based on UC, verifying the prevalence of HPV in immunosuppressed kidney transplant patients at a reference hospital.

MATERIAL AND METHOD

A cross-sectional study was carried out, in which the analyzed population was composed of kidney transplant patients from a hospital in Porto Alegre who underwent routine UC from August 2012 to August 2014. Patients older than 18 years, of both sexes, who signed the informed consent, participated in the study.

All the morning spontaneous urine specimens were collected in sterile containers. Each sample was processed in duplicate as follows: a portion of 10 ml urine was centrifuged at 1,300 revolutions per minute (rpm) for 10 min, and the pellet was cytocentrifuged (Cytospin) at 800 rpm for 6 min. Slides were stained with Papanicolaou method and visualized under an optical microscope at 200× and 400× magnification. The entire slide area (5 mm diameter) was analyzed.

Slides were assessed in a double-blind fashion by two experienced cytologists from the reference service. The adopted criterion for HPV detection was identification of superficial and intermediate cells with nuclear atypia, clear cytoplasmic halo, and thickening of the

peripheral membrane. The diagnosis of HPV infection was made by the presence of koilocytes in microscopic analysis.

Patient selection

Subjects were selected by convenience sampling. Kidney transplant patients were identified by the presence of HPV (koilocytes) in the mentioned exam. An interview was carried out and the study was explained for patients to sign the informed consent. At the end of this step, participants received printed material about prevention of cervical cancer supplied by the Ministry of Health (MS).

Data collection

Data from patients' records were used as collection instrument. The study followed the guidelines of Resolution nº 466/2012 of the National Council of Health/MS, and was approved by the ethics committee of Irmandade Santa Casa de Misericórdia de Porto Alegre (ISCOMPA). The researchers declare that there are no conflicts of interest. All the participants signed the informed consent.

Data analysis

All the UC results from kidney transplant patients in the mentioned period were analyzed. All patients under suspicion of HPV in the report had their slides revised by at least one of the researchers. The slides considered positive for HPV met the same cytopathologic screening criteria for diagnosis of cervical HPV, that is, cells had perinuclear halo. As quality control, after analysis by researchers, two experienced pathologists checked the diagnoses in a double-blind manner, and in case there were no discrepancies in the results, they were taken into consideration. Images of patients' UC slides were captured by means of an optical microscope connected with a camera and specific software.

Report data were collected, stored in an electronic spreadsheet and analyzed by statistical software. Correlation between variables was evaluated by means of *t* test with 5% ($p < 0.05$) statistical significance.

RESULTS

In the analyzed period, 2,305 UCs were performed to assess the presence of viral cytopathic effects (decoy cells – polyomavirus) in kidney transplant patients. Among this total, 13 cases detected

the presence of koilocytes in the UC test (**Figure**). The relative frequency of patients with the presence of HPV in urine was 0.56%.

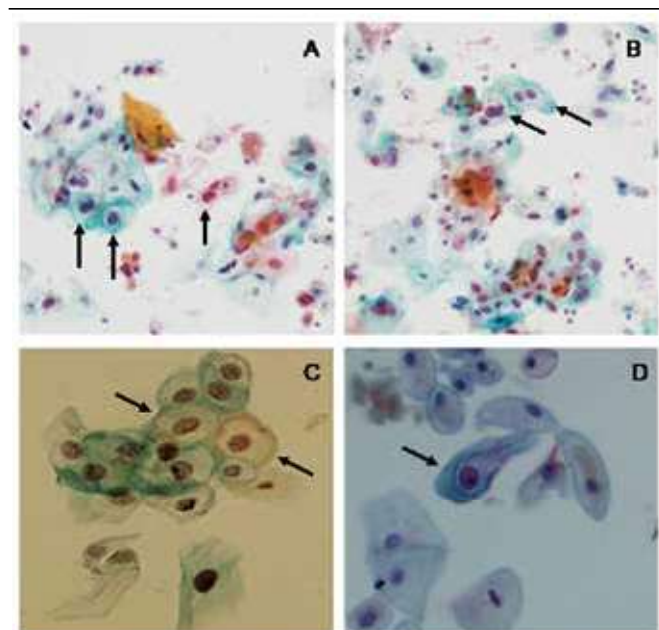


FIGURE — Presence of koilocytes in UC. A and B: 20×; C and D: 40×, optical microscopy, Papanicolaou staining. Images by Leon, RM
UC: urine cytology test.

Among the patients with HPV detected in urine ($n = 13$), 10 (76.9%) were females. The age group with the highest frequency was that of 51-70 years (53.8%), followed by those of 18-30 years (23.1%) and 31-50 years (23.1%).

Regarding the profile of patients as to pre-transplant sexually transmitted diseases (STDs) (**Table 1**), all presented human immunodeficiency virus (HIV)-negative serology and negative syphilis (Venereal Diseases Research Laboratory [VDRL]) test. Among the 13 patients, two (15.4%) were positive for hepatitis B virus (HBV), and four were positive for hepatitis C virus (HCV). HBV-HCV co-infection was observed in two (15.4%) patients.

The immunosuppressive regimen used in patients during the first month after transplantation consisted of tacrolimus (Tac), mycophenolate sodium (MPS), and prednisone (Pred). Dosage (**Table 1**) presented higher frequencies in the ranges of Tac 4.5-8 mg/day, corresponding to 54.5% of patients; MPS 1,081-1,440 mg/day in 63.6%; and Pred 18-20 mg/day in 72.7%.

Time between transplantation and the first UC with the presence of koilocytes (**Table 2**) was observed. One can note the highest frequencies occurred in up to six months after transplantation in six (46.15%) patients, and between six months and one year in four (30.77%) patients, summing up 76.92%.

TABLE 1 — Patients' profiles as to pre-transplant STDs and immune status at the first post-transplant visit

Variable	n (%)
Negative HIV and VDRL	13 (100)
Negative HBV	11 (84.6)
Positive HBV	2 (15.4)
Total	13 (100)
Negative HCV	9 (69.2)
Positive HCV	4 (30.8)
Total	13 (100)
Tac dose (mg/day)	
4.5-8	6 (54.5)
8.5-12	3 (27.3)
12.5-16	2 (18.2)
Total	11 (100)
MPS dose (mg/day)	
180-720	2 (18.2)
721-900	1 (9.1)
901-1,080	1 (9.1)
1,081-1,440	7 (63.6)
Total	11 (100)
Pred dose (mg/day)	
0	1 (9.1)
5-10	2 (18.2)
10.5-17.5	0 (0)
18-20	8 (72.7)
Total	11 (100)

STDs: sexually transmitted diseases; HIV: human immunodeficiency virus; VDRL: Venereal Diseases Research Laboratory; HBV: hepatitis B virus; HCV: hepatitis C virus; Tac: tacrolimus; MPS: mycophenolate sodium; Pred: prednisone.

TABLE 2 — Time between transplantation and the first UC with the presence of koilocytes

Time (months)	n (%)	(%) Accumulated
≤ 6	6 (46.15)	46.15
> 6 and < 12	4 (30.77)	76.92
> 12	3 (23.08)	100
Total	13 (100)	

UC: urine cytology test.

The percentage of women who underwent pre-transplant cervical cytology or anatomical pathology testing in the reference hospital was 30%: two with normal results and one (10%) with the presence of koilocytosis. After transplantation, cervical cytology was performed in three (30%) patients that presented alteration (low grade CIN [CIN I]). However, among the 10 women of the study, five (50%) did not undergo any pre- or post-transplant gynecological exam in the reference hospital. The use of oral contraceptives was observed in three (30%) patients of the study.

In the analysis of time between transplantation and the first UC with HPV cytopathic effect, 10 (76.92%) patients presented koilocytes ($p < 0.0001$) in the interval up to the first year after transplantation.

Dosages of immunosuppressant drugs, until the first post-transplant visit, which demonstrated correlation with the period between transplantation and the first UC with the presence of koilocytes ($p < 0.0001$) were Pred 10.5-20 mg/day, MPS 901-1,440 mg/day and Tac 4.5-12 mg/day.

DISCUSSION

Urine cytology test

UC is performed in all kidney transplant patients as a routine practice, and it can evidence the presence of the viral cytopathic effect. Considering that the presence of koilocytes is a pathognomonic sign of HPV infection⁽⁴⁾, UC has an adjuvant potential to screen for this infection. Although it is not specific for HPV detection, the relative frequency of kidney recipients' urine found positive for HPV (0.56%) demonstrates the possibility of detecting this virus by means of a low-cost non-invasive test. An interesting finding of the study was the presence of HPV in the urine of three male patients. The male population is known to not undergo routine HPV testing, thus they may infect their partners. Male partners can in fact contribute to the risk of developing cervical cancer in women, acting as "carriers" and "vectors" of HPV oncogenic types^(14,15). In the study by Antunes *et al.* (2004), the prevalence of koilocytosis in penile biopsies was 51.2% of 80 patients⁽¹⁴⁾.

As stated by Sousa *et al.* (2012), koilocytosis was found in 63% of cervical smears from women diagnosed as CIN I. This cytopathic effect was observed in 26.2% and 25.7% of smears from women diagnosed as CIN II and CIN III, respectively⁽¹⁶⁾. In a study with HIV-positive female patients, koilocytosis was found in 27.2% of women against 9.2% of HIV-negative women⁽¹⁷⁾. Among 33 patients with bladder carcinoma, koilocytosis was seen in tissue sections from 13 patients, and was observed in the UC of three. All the three were positive for high-risk HPV deoxyribonucleic acid (DNA); in conclusion, koilocytosis is a good morphological marker for HPV in the urothelium⁽¹⁸⁾.

Time after transplantation and presence of viral cytopathic effect in urine cytology

Time between transplantation and the first UC detecting koilocytosis, until six months and between six months and one

year, presented increased frequency with statistical significance ($p < 0.0001$). This finding for HPV is similar to that of other studied viruses according to the literature. In the study by Agrawal *et al.* (2010) with 327 kidney transplant patients during a period of four years, 13 patients were identified with kidney disease by polyomavirus; and four, by cytomegalovirus. All the patients were on a triple immunosuppressive regimen, with cyclosporine, Tac, and Pred or mycophenolate mofetil (MMF). The average time to diagnose viral infection after transplantation was 12.4 months for nephropathy by polyomavirus, and 4.8 months for nephritis by cytomegalovirus⁽¹⁹⁾.

Immunosuppressive doses and time between transplantation and urine cytology showing koilocytes

Infection in a kidney transplant recipient is an important cause of morbidity and mortality. It is many times detected late or remains undetected due to the impaired immune response caused by immunosuppressive therapy⁽¹⁹⁾.

In the present study, the doses of immunosuppressants up to the first visit after transplantation, which demonstrated correlation with the period between transplantation and the first UC showing koilocytes ($p < 0.0001$), were Pred 10.5-20 mg/day, MPS 901-1,440 mg/day, and Tac 4.5-12 mg/day. In this study, the higher the dose of immunosuppressants, the shorter the time between transplantation and the presence of koilocytes in the UC. Certain immunosuppressive agents, such as calcineurin inhibitors (Tac), and mycophenolate (antiproliferative), are associated with the increased risk of viral infections; the first year after transplantation corresponds to a period of intense immunosuppression in kidney transplant patients⁽²⁰⁾.

According to Cukuranovic *et al.* (2012), viruses are among the most common causes of post-transplant opportunistic infections, and many viral infections after transplantation result from the reactivation of a "latent" viral infection in the host. Several factors contribute to post-transplant viral activation, including immune suppression (especially reduction of cytotoxic immunity), graft rejection therapy, inflammation (cytokines) and tissue lesion. The intensity of immune suppression used to prevent graft rejection and other factors related to the host regulate susceptibility to viral infection⁽²¹⁾.

The importance of HPV screening in kidney transplant patients

The age group that presented the highest frequency of HPV presence in UC (51-70 years) comprises the period of higher risk to develop cervical cancer, between 40 and 60 years^(22,23).

Among the three patients that underwent pre-transplant cervical cytology test, two presented normal results; and one, alteration (koilocytosis). Among the three patients who underwent post-transplant cervical cytology test, all presented alteration. In the study by Paternoster *et al.* (2008), transplant patients underwent Papanicolaou test and HPV exams six months before and after transplantation. All of them had negative Pap smears before their grafts. After the procedure, 16 patients (10.59%) had negative Pap smears, but positive viral typing. Eleven (7.28%) presented positive Pap smears. The final incidence of HPV infection (15.23%) was consistent with the literature. The incidence of minor intraepithelial lesions of the female genital tract (7.28%) was higher than in the normal population and analogous studies (4.5%-8.5%). The study suggests beginning screening for HPV infection approximately six months before the graft to avoid an irreversible situation of difficult treatment⁽²⁴⁾.

Pre-transplant screening of recipients offers opportunity to determine prophylaxis and the prevention strategies adopted after transplantation, as well as to educate patients and their families on preventive measures⁽²¹⁾.

According to Oliveira *et al.* (2014), knowing the relationship between immunosuppression, viral infection and neoplasia,

it is necessary to close follow transplant patients, conducting periodical exams to early detect a possible cancer. In cases of persistent infections, this monitoring must be performed more frequently. Besides, depending on the type of immunosuppressant administered, whether potent or not, monitoring must be even more rigorous⁽²⁵⁾.

Quadrivalent HPV vaccine is recommended for post-transplant patients, as it is safe and well-tolerated⁽²⁶⁾. In the future, the inclusion of HPV vaccine in the immunization schedule for young adult patients in the kidney pre-transplant period is a strategy that may decrease the risk of developing cervical cancer in these patients.

CONCLUSION

Based on the results of our study, immunosuppression proved to be a major risk factor for HPV infection or HPV reactivation. Thus, gynecological follow-up must be part of pre- and post-transplant routine. Post-transplant UC screening, which is very important, can detect HPV infection. As immunosuppression is a risk factor for HPV infection, perhaps UC may be extended to other types of organ transplantation.

RESUMO

Introdução: O papilomavírus humano (HPV) é a principal causa de câncer de colo do útero, e a imunossupressão é reconhecida como fator de risco para infecção pelo HPV e sua persistência. Após o transplante renal, agentes imunossupressores são usados para evitar rejeição, mas predisõem o receptor a infecções crônicas e doenças malignas. **Objetivo:** Este trabalho teve como objetivo verificar, a partir do exame citológico urinário, a prevalência do HPV em pacientes transplantados renais imunossuprimidos. **Material e método:** Neste estudo transversal, a população foi composta por pacientes transplantados renais que fizeram o exame de rotina citológico urinário no período de agosto de 2012 a agosto de 2014. **Resultados:** Realizaram-se 2.305 exames citopatológicos de urina. Foram observados 13 pacientes com presença de coilocitos no referido exame. A frequência relativa de pacientes com HPV detectado na urina foi de 0,56%. No intervalo até o primeiro ano pós-transplante, 10 (76,92%) pacientes apresentaram coilocitos ($p < 0,0001$) no exame citológico urinário (ECU). As dosagens de imunossupressores até a primeira consulta pós-transplante, que demonstraram correlação com o período entre o transplante e o primeiro ECU com presença de coilocito ($p < 0,0001$), foram prednisona 10,5-20 mg/dia, micofenolato de sódio 901-1.440 mg/dia e tacrolimo 4,5-12 mg/dia. **Conclusão:** Este estudo mostrou a imunossupressão como um fator de risco importante para infecção pelo HPV ou sua reativação. O acompanhamento por meio do ECU pós-transplante pode evidenciar a infecção por HPV.

Unitermos: imunossupressão; transplante de rim; fatores de risco; infecções por papilomavírus.

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7.2 Parecer final orientadora estrangeira Nicola Annels

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16th March 2015

Re: Alana Ranzi

To whom it may concern:

Alana Ranzi worked with us in the Oncology group at the University of Surrey, UK from August 2014 until the end of January 2015. During her time with us Alana worked on a project evaluating the expression of a potential urinary diagnostic biomarker Engrailed-2 (EN2) by immunohistochemistry on three large independent cohorts of bladder cancer cases. Alana together with a pathologist at the Royal Surrey County Hospital scored all of the bladder cases from each of the cohorts for the intensity and localisation of EN2. Alana is currently finishing the statistical analysis of the scoring results to determine whether there is any correlation of EN2 expression with different stages and grades of disease and whether EN2 expression may be an early event in bladder tumourigenesis, and independent of the specific molecular dysregulation associated with NMIBC. Following completion of the statistical analysis we will together with Alana be preparing a manuscript for publication.

In addition to the immunohistochemical study outlined above, Alana also helped to set up a further study investigating the potential utility of urinary exosomal EN2 RNA as a biomarker in bladder cancer. For this project Alana set up exosome harvest from urines obtained from confirmed bladder cancer patients and patients seen at the haematuria clinic by filter column centrifugation and then performed subsequent RNA extraction using a commercially available kit. The exosomal RNA was then analysed using an Agilent Bioanalyzer which confirmed that Alana had successfully obtained exosomal RNA of acceptable quality from her samples. Alana then went on to perform amplification of the RNA and clean-up of the amplification products in preparation for EN2 PCR. Unfortunately due to her time in the UK coming to an end she was unable to perform the last step of EN2 PCR however, this will be completed by members of the Oncology group.

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Dr Agnieszka Michael, Senior Lecturer, Consultant in Medical Oncology
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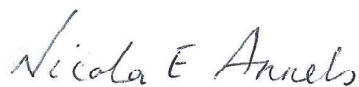
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Evaluation of Alana Ranzi

During her time with us Alana proved herself to be a highly competent, hard-working and dedicated student. I was extremely impressed by her ability to learn new techniques quickly and perform them independently. Not only did she complete the project she came to the UK to perform but she also helped set up an additional project with techniques that were new to our lab as well. Alana constantly worked hard and efficiently recorded and analysed her results. She became a very popular member of our group and as well as interacting well with members of the oncology group she also regularly met with a pathologist at the Royal Surrey County Hospital. It was a pleasure to host Alana in our group and I very much hope that we might have the opportunity to work together again in the future.

Dr Nicola Annels



Senior Research Fellow
Oncology

Prof Hardev Pandha, Consultant in Medical Oncology
Dr Agnieszka Michael, Senior Lecturer, Consultant in Medical Oncology
Immunology
Dr Alex Stewart, Senior Fellow, Consultant in Clinical Oncology
Fellow

Dr Richard Morgan, Senior Lecturer
Dr Nicola Annels, Senior Fellow in

Dr Kate Relph, Post Doctoral Research

Dr Guy Simpson, Post Doctoral Research

7.3 Parecer de aprovação CEP UFCSPA

UNIVERSIDADE FEDERAL DE
CIÊNCIAS DA SAÚDE DE
PORTO ALEGRE



PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Associação entre o estresse oxidativo e o câncer de bexiga.

Pesquisador: Claudia Giuliano Bica

Área Temática:

Versão: 2

CAAE: 07321012.9.0000.5335

Instituição Proponente: IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE

Patrocinador Principal: Universidade Federal de Ciências da Saúde de Porto Alegre

DADOS DO PARECER

Número do Parecer: 258.491

Data da Relatoria: 18/04/2013

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Projeto aprovado no CEP da Santa Casa. Os itens apresentados são considerações que devem ser revisados como sugestões pelos autores do estudo.

De acordo com o Parecer do Relator.

PORTO ALEGRE, 29 de Abril de 2013

Assinador por:

José Geraldo Vernet Taborda
(Coordenador)

7.4 Parecer de aprovação CEP ISCMPA

IRMANDADE DA SANTA CASA
DE MISERICORDIA DE PORTO
ALEGRE - ISCMPA



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Associação entre o estresse oxidativo e o câncer de bexiga.

Pesquisador: Claudia Giuliano Bica

Área Temática:

Versão: 2

CAAE: 07321012.9.0000.5335

Instituição Proponente: IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE

Patrocinador Principal: Universidade Federal de Ciências da Saúde de Porto Alegre

DADOS DO PARECER

Número do Parecer: 226.953

Data da Relatoria: 14/03/2013

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Após reavaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer.

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA. Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 - Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

PORTO ALEGRE, 22 de Março de 2013

Assinador por:
ELIZETE KEITEL
(Coordenador)

7.5 Normas revista *Pathology and Oncology Research*

Instructions for authors

Language: We appreciate any efforts that you make to ensure that the language is corrected before submission. This will greatly improve the legibility of your paper if English is not your first language.

Types of papers: The journal publishes Original Articles, Reviews, Short Communications and Letters to the Editor.

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Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

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The title page should include: The name(s) of the author(s), a concise and informative title, the affiliation(s) and address(es) of the author(s), the e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.

- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

Headings: please use no more than three levels of displayed headings.

Abbreviations: Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments: Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Scientific style: Please always use internationally accepted signs and symbols for units (SI units).

References

Citation: Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list: The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article
Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol* 105:731-738. doi: 10.1007/s00421-008-0955-8
Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:
- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med*. doi:10.1007/s001090000086

- Book
South J, Blass B (2001) The future of modern genomics. Blackwell, London
- Book chapter
Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document
Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. <http://physicsweb.org/articles/news/11/6/16/1>. Accessed 26 June 2007
- Dissertation
Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- ISSN.org LTWA

Tables: All tables are to be numbered using Arabic numerals, tables should always be cited in text in consecutive numerical order, for each table, please supply a table caption (title) explaining the components of the table, identify any previously published material by giving the original source in the form of a reference at the end of the table caption, footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

Electronic Figure Submission: Supply all figures electronically, indicate what graphics program was used to create the artwork, for vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable, vector graphics containing fonts must have the fonts embedded in the files, name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art: Definition: Black and white graphic with no shading, do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size, all lines should be at least 0.1 mm (0.3 pt) wide, scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi, vector graphics containing fonts must have the fonts embedded in the files.

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Color Art: color art is free of charge for online publication, if black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent, if the figures will be printed in black and white, do not refer to color in the captions, color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering: To add lettering, it is best to use Helvetica or Arial (sans serif fonts), keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt), variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label, avoid effects such as shading, outline letters, etc, do not include titles or captions within your illustrations.

Figure Numbering: All figures are to be numbered using Arabic numerals, figures should always be cited in text in consecutive numerical order, figure parts should be denoted by lowercase letters (a, b, c, etc.), if an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions: Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file, figure captions begin with the term **Fig.** in bold type, followed by the figure number, also in bold type, no punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption, identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs, identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size: Figures should be submitted separately from the text, if possible, when preparing your figures, size figures to fit in the column width, for most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm, for books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.

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- Informed consent

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- Position on advisory board or board of directors or other type of management relationships
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The corresponding author will include a summary statement in the text of the manuscript in a separate section before the reference list, that reflects what is recorded in the potential conflict of interest disclosure form(s).

See below examples of disclosures:

Funding: This study was funded by X (grant number X).

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Conflict of Interest: The authors declare that they have no conflict of interest.

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1) Statement of human rights

When reporting studies that involve human participants, authors should include a statement that the studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that the independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study.

The following statements should be included in the text before the References section:

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For retrospective studies, please add the following sentence:

"For this type of study formal consent is not required."

Informed consent

All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. Hence it is important that

all participants gave their informed consent in writing prior to inclusion in the study. Identifying details (names, dates of birth, identity numbers and other information) of the participants that were studied should not be published in written descriptions, photographs, and genetic profiles unless the information is essential for scientific purposes and the participant (or parent or guardian if the participant is incapable) gave written informed consent for publication. Complete anonymity is difficult to achieve in some cases, and informed consent should be obtained if there is any doubt. For example, masking the eye region in photographs of participants is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic profiles, authors should provide assurance that alterations do not distort scientific meaning.

The following statement should be included:

Informed consent: "Informed consent was obtained from all individual participants included in the study."

If identifying information about participants is available in the article, the following statement should be included:

"Additional informed consent was obtained from all individual participants for whom identifying information is included in this article."

7.6 Normas revista *Journal of Biomedical Science*

Research article

Preparing your manuscript

The Title page should:

- present a title that includes, if appropriate, the study design e.g.:
 - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
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- list the full names, institutional addresses and email addresses for all authors
 - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the "Acknowledgements" section in accordance with the instructions below
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used
- **Results:** the main findings
- **Conclusions:** brief summary and potential implications
- **Trial registration:** If your article is a systematic review or reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be stated in this section. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

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If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

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- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

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For all journals, authors must include an “Availability of data and materials” section in their article detailing where the data supporting their findings can be

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Competing interests

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Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our [editorial policies](#).

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

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Group authorship: if you would like the names of the individual members of a collaboration Group to be searchable through their individual PubMed records, please ensure that the title of the collaboration Group is included on the title page and in the submission system and also include collaborating author names as the last paragraph of the “Acknowledgements” section. Please add authors in the format First Name, Middle initial(s) (optional), Last Name. You can add institution or country information for each author if you wish, but this should be consistent across all authors.

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Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

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Illustrations and figures (if any)

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