U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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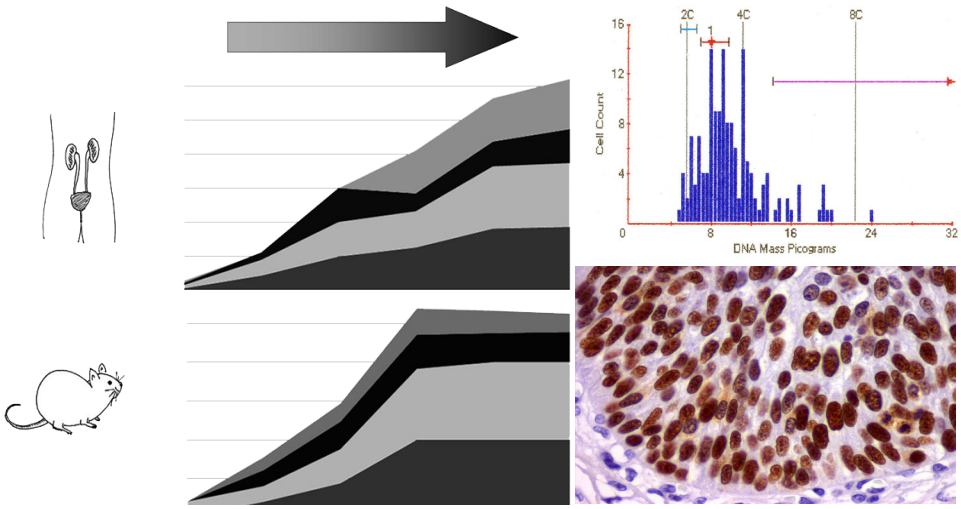
ESTUDO COMPARATIVO ENTRE O CARCINOMA UROTELIAL DA BEXIGA NO HOMEM E O CARCINOMA UROTELIAL INDUZIDO QUIMICAMENTE EM ROEDORES (MURGANHO E RATO)



CARLOS ALBERTO PALMEIRA DE SOUSA

Tese de doutoramento em Ciências Biomédicas

2010

TÍTULO Estudo comparativo entre o carcinoma uroterial da bexiga no Homem e o carcinoma uroterial induzido quimicamente em roedores (murganho e rato)

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EDIÇÃO Do Autor

ISBN 978-989-20-2286 - 9

ANO 2011

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

À minha esposa e aos meus filhos

AGRADECIMENTOS

O conjunto de trabalhos aqui reunidos e que constituem esta tese de doutoramento, contou com a contribuição directa ou indirecta de uma extensa lista de pessoas, às quais desejo manifestar o meu mais profundo e sincero agradecimento. Não posso deixar, no entanto, de citar algumas pela contribuição decisiva que tiveram.

Não respeitando, talvez, as questões de protocolo, queria começar pela minha esposa e pelos meus filhos. Foram os mais afectados pelas minhas "ausências", retribuindo sempre com enorme paciência e muito ânimo e confiança. Muito obrigado.

Ao Professor Doutor Lúcio Lara Santos, principal orientador científico desta tese de doutoramento, o meu agradecimento e reconhecimento pelo encorajamento e instigação constantes, pela enorme disponibilidade e capacidade de trabalho, pela amizade, não só ao longo da elaboração desta tese, mas em muitos outros trabalhos em conjunto.

Ao Professor Doutor Carlos Lopes, o meu mais profundo sentimento de gratidão pelo encaminhamento científico e profissional, pelos valiosos ensinamentos que sempre me transmitiu, tão fundamentais desde o meu primeiro dia no Instituto Português de Oncologia do Porto.

À Professora Doutora Paula Oliveira, co-orientadora científica desta tese, quero agradecer a sua inexcedível motivação, capacidade de trabalho e preciosa contribuição e colaboração científica e pessoal.

À Dra. Catarina Lameiras, agradeço a partilha de dados e os enriquecedores "brainstormings" realizados.

Por último, mas não menos importantes, quero agradecer aos meus pais e irmã (longe, mas sempre presentes), aos meus sogros e restante família, aos meus "colegas" de laboratório, pelo apoio e confiança sempre constantes.

ABREVIATURAS UTILIZADAS NO TEXTO

ADN	Ácido desoxirribonucleico
BBN	N-butil-N-(4-hidroxibutil) nitrosamina
BCG	*Bacillus* Calmette Guerin
CIS	carcinoma *in situ*
CR	cistectomia radical
CUB	carcinomas uroteliais da bexiga
DNA	"deoxyribonucleic acid"
EGFR	"epidermal growth factor receptor"
EORTC	"European Organization for Research and Treatment of Cancer"
HER2	"human epidermal growth factor receptor"
IHQ	imunohistoquímica
IPOPFG-EPE	Instituto Português de Oncologia do Porto Francisco Gentil-EPE
LOH	perda de heterozigotia
mTOR	"mammalian target of rapamycin"
PTEN	"phosphatase and tensin homolog"
RTU	ressecção transuretral
VEGF	"vascular endothelial growth factor"

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RESUMO

O cancro da bexiga continua a ser um grave problema de saúde pública em todo o mundo, constituindo o quinto tipo de cancro mais frequente nos países industrializados.

Os protocolos terapêuticos correntes não conseguem responder com eficácia a todos os grupos de doentes com carcinoma urotelial da bexiga (CUB). Embora a elevada taxa de recorrência dos CUB superficiais constitua um importante desafio terapêutico, é ao nível dos CUB invasores que maior necessidade há de mais e melhor investigação, visto serem os principais responsáveis pela morbilidade e mortalidade devida a este tipo de cancro.

No processo de investigação e avaliação de novas estratégias terapêuticas para o cancro da bexiga, tem desempenhado um papel fundamental a utilização de modelos animais, que permitem, por um lado, o ensaio pré-clínico dos novos agentes terapêuticos e, por outro, um conhecimento mais profundo do processo de carcinogénese urotelial da bexiga.

O presente conjunto de trabalhos teve como objectivo geral a comparação entre as lesões uroteliais da bexiga induzidas quimicamente em roedores, com o carcinogénio N-butil-N-(4-hidroxibutil) nitrosamina (BBN), e as lesões observadas no modelo de carcinogénese humana. Foram estudadas amostras de bexiga de murganho, rato e de uma série de peças de cistectomia de doentes humanos com cancro da bexiga. Para além da caracterização histopatológica das lesões uroteliais quimicamente induzidas e das lesões uroteliais do modelo humano, procedeu-se à caracterização biológica das lesões através do estudo de três variáveis com importância reconhecida ao nível do humano: conteúdo de ADN por citometria de imagem, imunoexpressão da proteína p53 por imunohistoquímica (IHQ) e índice proliferativo mediante a imunoexpressão da proteína Ki-67, também por IHQ. No modelo experimental, os animais foram divididos em grupos, sujeitos a diferentes condições de exposição à BBN (tempo

de exposição), e tratamento com mitomicina C (MMC) ou *Bacillus* Calmette-Guerin (BCG), de acordo com o desenho experimental de cada trabalho.

Inicialmente observámos ser necessário um tempo mínimo de 20 semanas de exposição à BBN para a indução de CUB, particularmente os superficiais de alto grau e os invasores. O desenvolvimento das lesões uroteliais nos roedores, verificou-se no espaço intravesical, confirmando o carácter ortotópico deste modelo animal, tendo-se observado todo o espectro de lesões uroteliais descrito para o modelo de carcinogénese humana.

As lesões observadas nos dois modelos foram sobreponíveis, quer em termos histológicos, quer em termos biológicos, ou seja, o perfil de alterações biológicas foi idêntico em ambos os modelos: as frequências de aneuploidia de ADN, de sobreexpressão da p53 e elevação do índice proliferativo avaliado pela Ki-67, aumentaram com a agressividade das lesões uroteliais.

Os animais tratados com MMC e BCG revelaram menor frequência de CUB, como esperado. No entanto, os tumores invasores que persistiram após estes tratamentos, ou seja, aparentemente resistentes à terapia ao agente alquilante (MMC) e ao agente imunoterápico (BCG), apresentaram índices de proliferação mais baixos. Também no modelo humano observamos este subgrupo de invasores que, apesar de aneuplóides, revelaram menor taxa de proliferação. Estes resultados sugerem a existência de um subgrupo de CUB invasores potencialmente resistentes a algumas das terapias correntes, para os quais, se deverá, no futuro, avaliar qual a terapia mais eficaz.

Os CUB superficiais de alto grau com CIS concomitante, são outro grupo cujo tratamento suscita intenso debate. Na tentativa de contribuir para um maior esclarecimento sobre o significado da detecção de uma lesão de CIS na mucosa urotelial humana, verificámos que o CIS apresentava um perfil biológico característico: aneuploidia, aumento da expressão da p53 e índice proliferativo aumentado. Constatámos, ainda, que este perfil era constante, independentemente da localização do CIS na mucosa (adjacente ou à distância do tumor) e do tipo de tumor associado (CUB superficial de alto grau ou invasor). Observámos, também, que, sempre que esta lesão estava presente, a mucosa urotelial aparentemente normal apresentava o mesmo perfil de alterações biológicas do CIS: Estes resultados sugerem, por um lado, a existência de uma

assinatura genética característica do CIS e, por outro, que a detecção de CIS na mucosa urotelial é um marcador de alarme para a existência de uma intensa instabilidade genética generalizada a todo o urotélio, e do processo de cancerização em campo. O tratamento mais adequado a estes doentes deverá ter, em linha de conta, este carácter de doença "em campo". Os CIS detectados nos roedores, após instilações repetidas de MMC, com ou sem indução prévia pela BBN, eram biologicamente semelhantes aos do modelo humano

Apesar das semelhanças verificadas entre os dois modelos de carcinogénese urotelial da bexiga aqui testados, observamos que as alterações biológicas características dos CUB invasores no homem foram detectadas em lesões mais iniciais no modelo animal. Estes resultados sugerem que este modelo animal deverá ser mais adequado para o estudo dos CUB mais avançados, os superficiais de alto grau e os invasores e mestastáticos, principais responsáveis pela mortalidade por cancro da bexiga, como atrás referido.

SUMMARY

Bladder cancer remains a serious public health problem throughout the world, being the fifth most common type of cancer in industrialized countries.

The current available therapeutic protocols fail to respond effectively to all groups of patients with urothelial carcinoma of the bladder. Although frequent recurrences in superficial urothelial carcinoma place a therapeutic challenge, invasive tumours represent the major cause of morbidity and mortality and, therefore, more therapeutic research studies should be performed regarding this bladder cancer group. In this process of research, the use of animal models has been important to the pre-clinical development of new effective therapeutic agents and to enable a deeper and accurate understanding of the bladder carcinogenesis process.

The main goal of the present set of studies was to compare the bladder urothelial lesions chemical induced in rodents, with the carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), and the urothelial lesions observed in the human carcinogenesis model We studied samples of bladder mice, rats and a number of human cystectomy specimens from patients with bladder cancer. In addition to the histopathologic characterization, we performed the biological evaluation of the urothelial lesions by studying three variables with acknowledged importance in human model: DNA content by image cytometry, immunoexpression of p53 protein by immunohistochemistry and proliferative index by immunoexpression of Ki-67 protein, also by immunohistochemistry. In the experimental model, the animals were divided into groups under different conditions of exposure to BBN (exposure time), and treatment with mitomycin C (MMC) or *Bacillus* Calmette-Guerin (BCG), according to the experimental design of each work.

Initially, we found that a minimum of 20 weeks of exposure to BBN was necessary for the induction of urothelial carcinomas, particularly superficial high-grade and invasive tumours. The development of the urothelial lesions in rodents occurs in the intravesical space, confirming the orthotopic nature of this animal model, and it was observed all the urothelial lesions described for the human bladder carcinogenesis model.

The lesions observed in the two models were superimposed, both in terms of histopathology and biological features, this is, the alterations in the biological profile were identical in both models: the frequencies of DNA aneuploidy, over-expression of p53 and high proliferative index assessed by Ki-67, increased with the aggressiveness of the urothelial lesions.

Animals treated with MMC and BCG showed lower frequency of urothelial carcinomas, as expected. However, the invasive tumours that remains after these treatments, apparently resistant to the used therapeutic alkylating (MMC) and immunotherapeutic (BCG) agents, showed lower proliferation index. This subgroup of invasive tumours, with aneuploid DNA content but low rate of proliferation, was also observed in human model. These results suggest the existence of a subgroup of invasive urothelial carcinomas, potentially resistant to some of the current therapies, for which it should be found the most effective therapy.

The superficial high grade tumours with concomitant carcinoma *in situ* (CIS), are another group of tumours whose treatment gives rise to intense debate. In an attempt to contribute to the further clarification of the significance of the detection of CIS lesions in human urothelial mucosa, we found that the CIS had a distinctive biological profile: aneuploidy, increased p53 expression and increased proliferative rate. We also noted that this profile was constant regardless of the location of the CIS in the mucosa (adjacent to or away from the tumour) and the type of associated tumour (superficial high-grade urothelial carcinoma or invasive tumour). It was also observed that, when this lesion was present, the apparently normal urothelial mucosa showed the same pattern of biological changes of the CIS. These results suggest, first, the existence of a genetic signature characteristic of CIS and, secondly, that the detection of CIS is a surrogate marker for a marked genetic instability widespread throughout the urothelium, and the

process of field cancerization. The most appropriate treatment for these patients should include a “field treatment" approach. In rodents, CIS was detected after repeated instillations of MMC, with or without prior induction by BBN, and it was biologically similar to those observed in human model

The similarities found between these two models of bladder urothelial carcinogenesis tested here, in humans and rodents, allow taking the validated animal model for the study of high grade superficial urothelial tumours and invasive carcinomas.

LISTA DE PUBLICAÇÕES

Os artigos a seguir discriminados foram elaborados e publicados como parte da presente Tese Doutoral, sendo numerados de acordo com a sua apresentação no texto:

Modelo Humano

Artigo #1: Palmeira CA, Oliveira PA, Seixas F, Pires MA, Lopes C, Santos L. DNA image cytometry in bladder cancer: State of the art. Anticancer Res. 2008; 28: 443-450.

Artigo #2: Santos L, Lameiras C, Afonso J, Palmeira C, Pereira S, Costa C, Amaro T, Bento MJ, Morais A, Criado B, Lopes C. Is DNA content alteration a consequence of proliferative and differentiation changens in urothelial bladder tumours? Acta Urol 2003; 20: 9-17.

Artigo #3: Palmeira C, Lameiras C, Amaro T, Lima L, Kock A, Lopes C, Oliveira PA, Santos L. CIS is a surrogate marker of genetic instability and field carcinogenesis in the urothelial mucosa. Urol Oncol 2009 (em publicação).

Modelo Animal

Artigo #4: Oliveira PA, Palmeira C, Lourenço LM, Lopes CA. Evaluation of DNA content in preneoplastic changes of mouse urinary bladder induced by N-butyl-N-(4-hydroxybutyl) nitrosamine. J Exp Clin Cancer Res. 2005; 24: 609-616.

Artigo #5: Palmeira C, Oliveira PA, Arantes-Rodrigues R, Colaço A, De la Cruz PL, Lopes C, Santos L. DNA cytometry and kinetics of rat urothelial lesions during chemical carcinogenesis. Oncol Rep. 2009; 21: 247-252.

Artigo #6: Oliveira PA, Palmeira C, Colaço A, de la Cruz LF, Lopes C. DNA content analysis, expression of Ki-67 and p53 in rat urothelial lesions induced by N-butyl-N-(4-hydroxybutyl) nitrosamine and treated with mitomycin C and bacillus Calmette-Guérin. Anticancer Res. 2006; 26: 2995-3004.

Artigo #7: Oliveira PA, Adega F, Palmeira CA, Chaves RM, Colaço AA, Guedes-Pinto H, De la Cruz P LF, Lopes CA. DNA study of bladder papillary tumours chemically induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in Fisher rats. Int J Exp Pathol. 2007; 88: 39-46.

Artigo #8: Oliveira P, Palmeira C, Colaço A, De la Cruz P LF, Lopes C. Cell proliferation and DNA content in rat urothelial lesions after repeat intravesical instillations of mitomycin C and bacillus Calmette-Guérin. Urol Int. 2008; 80: 90-97.

Comparação entre os modelos humano e animal

Artigo #9: Palmeira C, Oliveira P, Lameiras C, Amaro T, Silva VM, Lopes C, Santos L. Biological similarities between chemical and natural human bladder carcinogenesis. Oncol Letters. Oncology Letters 2010; 1 (2): 373-377.

INTRODUÇÃO

O cancro da bexiga é o segundo tipo de cancro mais frequente do tracto genito-urinário [Williams e Stein, 2004] e o quinto mais frequente nos países industrializados [Jemal et al., 2008], constituindo, por isso, um grave problema de saúde pública em todo o mundo. A sua incidência tem vindo a aumentar, registando-se 359557 novos casos em 2002 [Ferlay, 2004].

Em Portugal registaram-se 1631 novos casos em 2001 [Registo Oncológico Nacional, 2001]. No Norte do país são diagnosticados 631 novos casos de tumores da bexiga por ano (taxa de incidência bruta de 19,2), dos quais cerca de 22% são tratados no Instituto Português de Oncologia do Porto (IPOPFG-EPE) [RORENO, 2005]. Em 2007, foram admitidos e tratados neste Centro 170 novos casos de tumores da bexiga: 96 (56,5%) eram tumores uroteliais superficiais, 69 (40,6%) tumores uroteliais invasores e 5 (2,9%) eram tumores não uroteliais, de acordo com o estudo histológico. Do grupo de tumores uroteliais invasores 35 (50,7%) casos apresentavam metastização local ou à distância [Registo Oncológico, 2007].

A incidência deste tipo de cancro aumenta com a idade, revelando um pico entre os 50 e os 70 anos, sendo considerado mais comum nos homens que nas mulheres (a razão mundial é de cerca de 3,5:1) [Lopez-Beltran, 2004]

Nos países industrializados os factores de risco associados a este tipo de cancro incluem o fumo do tabaco, a ingestão de arsénio e a exposição a aminas aromáticas, agentes alquilantes e tratamentos de radioterapia [Cheng, 2008; Lopez-Beltran, 2004]. Nos países menos desenvolvidos considera-se, ainda, a infecção crónica pelo *Schistosoma sp*. um factor de risco associado a esta neoplasia [Lopez-Beltran, 2004].

Mais de 90% dos cancros da bexiga diagnosticados nos países chamados desenvolvidos, derivam do epitélio urotelial, ou seja, são carcinomas uroteliais da bexiga (CUB) [Fleshner, 1996]. A maioria destes doentes (70-80%) são diagnosticados com tumores papilares de baixo grau, superficiais, ou seja não invasores da camada muscular [Lopez-Beltran, 2004], e apresentam baixa tendência para progressão (10-20%), apesar do risco de recorrência ser alto (50-

70%) [Kaufman, 2009]. Os restantes doentes com CUB (cerca de 30%) apresentam-se ao diagnóstico com tumores de alto grau, invasores ou com doença metastática. A significativa redução na sobrevivência destes doentes, especialmente devido à presença de metástases, faz com que este grupo de tumores seja o principal responsável pela mortalidade por cancro da bexiga. A taxa de sobrevivência aos 5 anos nos doentes com tumores da bexiga admitidos no IPOPFG-EPE entre 2000 e 2001 foi de 60% [Registo Oncológico, 2001].

É por todos aceite que os CUB apresentam uma profunda heterogeneidade, quer histopatológica, quer em termos de resposta à terapia e sobrevivência. Esta heterogeneidade tem motivado uma intensa investigação ao nível das estratégias terapêuticas mais adequadas a estes doentes [Kaufman, 2009]. Apesar dos avanços alcançados e das recomendações estabelecidas na prática clínica, para cada um dos grupos de CUB, existem ainda alguns subgrupos de doentes, para os quais persiste uma intensa controvérsia sobre o esquema terapêutico mais eficaz.

Em relação aos CUB superficiais, a principal questão em discussão refere-se à definição do tratamento dos doentes com alto risco de recorrência e progressão para um estádio de doença invasora da camada muscular. De acordo com a European Organization for Research and Treatment of Cancer (EORTC), os factores de risco que definem estes doentes são: multifocalidade, tamanho do tumor, estádio, frequência de recorrência após ressecção transuretral (RTU), presença de carcinoma *in situ* (CIS) concomitante e grau tumoral [Babjuk, 2008]. Apesar do tratamento intravesical com o imunomodulador *Bacillus* Calmette-Guerin (BCG) em esquemas de manutenção ser o tratamento de referência [Jakse, 2001; Losa, 2000], diversos estudos referem a vantagem do recurso à cistectomia radical (CR) em alguns destes doentes, antes da progressão para doença invasora [Babjuk, 2008; Kaufman, 2009; Kulkarni, 2007].

Os CUB invasores, como atrás foi referido, representam a principal causa de morbilidade e mortalidade por cancro da bexiga [Youssef, 2009], e, por isso, a escolha do tipo de tratamento mais adequado para estes tumores suscita um debate intenso. A CR é, até o momento, o tratamento preconizado para estes doentes, embora diversos ensaios com quimioterapia neoadjuvante e adjuvante e

abordagens que permitem preservar a bexiga, estejam a ser investigados (105, 107, [Evans, 2009; Kaufman, 2009; Youssef, 2009]. Apesar da contínua optimização terapêutica, aproximadamente 50% destes doentes acabam por recorrer e morrer devido à doença [Rosenberg, 2009]. A maioria dos que recorrem após CR, apresentam metástases à distância, factor decisivo para a redução da sua sobrevivência [Rosenberg, 2009]. Contribuindo para esta indefinição, existem ainda tumores invasores com características biológicas distintas, que exigem abordagens terapêuticas igualmente específicas, como os tumores invasores com índices proliferativos baixos [Grossman, 2006; Santos, 2003a] ou com alterações moleculares, por exemplo, ao nível da expressão de "epidermal growth factor receptor" (EGFR), "human epidermal growth factor receptor" (HER2) e "vascular endothelial growth factor" (VEGF) [Kaufman, 2009].

Nos últimos anos têm sido investigados novos factores, nomeadamente variáveis biológicas, que permitam, a par dos factores histopatológicos convencionais, estratificar os doentes para melhor seleccionar o tratamento mais eficaz. Para tal, é necessário conhecer, de forma mais exacta, os mecanismos biológicos fundamentais para os processos de carcinogénese e progressão dos CUB, em particular dos tumores mais agressivos. Nesta investigação tem sido fundamental o desenvolvimento de diferentes modelos animais experimentais, que simulem o modelo humano em termos histológicos, características genéticas e bioquímicas, história natural e comportamento biológico [Oliveira, 2006a].

Alterações do gene *TP53*

Diversos grupos de investigação têm avaliado as alterações do gene *TP53* no cancro da bexiga, nomeadamente através de estudos de perda de heterozigotia (LOH) do cromossoma 17p, análise de mutações pontuais do gene *TP53* e avaliação dos níveis de expressão da proteína p53 [Cordon-Cardo, 2008]. Estas alterações têm sido associadas com o estádio e grau tumoral, sendo documentadas com maior frequência nos CIS e CUB invasores da camada muscular [Rosenberg, 2009]. Para além desta associação com os tumores invasores, quer em humanos [Cordon-Cardo, 2008], quer em ratos [Oliveira, 2006a; Puzio-Kuter, 2009], tem revelado uma associação significativa com o prognóstico destes tumores [Cordon-Cardo, 2008; Lacy, 2009; Santos, 2003a], permitindo, em conjugação com a perda de outros genes supressores tumorais,

como o "phosphatase and tensin homolog" (PTEN), identificar um subgrupo de doentes particularmente agressivos [Puzio-Kuter, 2009].

Alterações do índice proliferativo

As alterações na proliferação celular, decorrentes da perda do controlo do ciclo celular, têm sido documentadas e estudadas nos CUB [Grossman, 2006; Margulis, 2009; Santos, 2003a]. O estudo da expressão da proteína Ki-67 tem sido um dos marcadores mais utilizados para avaliar estas alterações do índice proliferativo nestes tumores. A adição deste marcador a outros factores de prognóstico estabelecidos, segundo alguns estudos, permite aumentar a capacidade de identificar subgrupos de doentes com maior risco de progressão, quer ao nível dos CUB superficiais, quer ao nível dos invasores. De acordo com estes resultados, estes doentes poderiam beneficiar de terapias adjuvantes [Margulis, 2009; Santos, 2003a].

Alterações do conteúdo de ADN

Na literatura sobre cancro da bexiga, é possível encontrar um vasto número de trabalhos científicos que procuraram definir a utilidade da avaliação do conteúdo de ADN na prática clínica, ao nível do rastreio, do prognóstico e da detecção de recorrência [Bol, 2003; Planz, 2000; Richman, 1998; van Velthoven, 2000; Wheeless, 1993]. Para além do seu valor de prognóstico, em especial quando avaliado em associação com outros parâmetros biológicos (índice proliferativo, variáveis morfométricas, etc), o conteúdo de ADN constitui um valioso instrumento de estudo do percurso biológico associado a cada tipo de tumor. A história biológica do cancro da bexiga revela que a aneuploidia de ADN surge pela crescente instabilidade genética e acumulação de sucessivas alterações genéticas, ao longo do processo de carcinogénese, ocorrendo com grande frequência nos CUB mais avançados [Wheeless, 1993]. Esta avaliação global das alterações genéticas pode ser muito útil quando se pretende comparar modelos de carcinogénese, nomeadamente as lesões uroteliais observadas no modelo animal e as suas correspondentes no modelo humano.

Modelos animais

A utilização de modelos animais continua a ter um papel fundamental na investigação pré-clinica de novas opções terapêuticas para o cancro da bexiga, nomeadamente na avaliação de novos agentes quimioterápicos e imunoterápicos, novas doses e esquemas terapêuticos e outras modalidades anti-tumorais, como as terapias alvo [Arentsen, 2009; Kaufman, 2009].

Pelo facto da carcinogénese química ter um papel fundamental no desenvolvimento do cancro da bexiga humano, um dos modelos animais mais utilizados é a indução química de cancro da bexiga em roedores. A indução experimental de cancro da bexiga em animais de laboratório pode ser conseguida mediante a utilização de três compostos carcinogénicos químicos genotóxicos diferentes: a N-metil-N-nitrosureia, a N-[4-(5-nitro-2-furil)-2-tiazolil] formamida e a N-butil-N-(4-hidroxibutil) nitrosamina (BBN) [Oliveira, 2006a]. Por questões de segurança e funcionalidade a BBN é o carcinogénio mais utilizado e induz o crescimento de tumores da bexiga no espaço intravesical (ortotópico).

Um dos desafios mais importantes na validação destes modelos experimentais é o estabelecimento de um modelo reprodutível e que simule o modelo humano nos aspectos histopatológicos, genéticos e comportamento biológico.

Motivação e Objectivos

Pelo atrás referido, podemos constatar que persiste a necessidade de se definir o tratamento mais eficaz nos grupos de doentes com CUB que mais contribuem para a mortalidade devida a este tipo de cancro, nomeadamente os CUB superficiais de alto grau e CUB invasores e metastáticos.

Apesar de se ter comprovado que o carcinoma urotelial é sensível a várias drogas citotóxicas o seu impacto em termos de controlo da doença metastática e sobrevivência é mitigado, pelo que não está, ainda, definido os protocolos terapêuticos com recurso a drogas citotóxicas e/ou biológicas adequados para este tipo de neoplasia, como ocorre em outros tumores.

Assim, o papel da quimioterapia neoadjuvante e adjuvante, é, ainda, fonte de controvérsia e de investigação [Kaufman, 2009]. A melhoria da sobrevivência global com os esquemas terapêuticos correntes traduziu-se, apenas, num aumento de 5-7% aos 5 anos [Stenzl, 2009].

Deste modo é pertinente conhecer os aspectos biológicos e patológicos que envolvem o processo de carcinogénese urotelial da bexiga e a progressão da doença, no sentido de se identificar novos alvos terapêuticos, novos agentes e novas estratégias terapêuticas. Porém, os doentes com CUB são maioritariamente idosos e podem ser portadores de patologias associadas, o que limita os estudos clínicos. Deste modo, os modelos experimentais *in vivo* têm um papel fundamental no esclarecimento dos problemas referenciados anteriormente.

O objectivo geral desta Tese Doutoral consistiu em comparar as características histológicas e biológicas dos carcinomas uroteliais da bexiga induzidos quimicamente em roedores, com os carcinomas uroteliais da bexiga humanos.

De forma mais específica, pretendeu-se:

- Identificar as lesões uroteliais benignas e malignas resultantes da carcinogénese quimicamente induzida em roedores

- Comparar as características histológicas entre as lesões uroteliais quimicamente induzidas em roedores e as lesões uroteliais do modelo humano

- Comparar as características biológicas entre as lesões uroteliais quimicamente induzidas em roedores e as lesões uroteliais do modelo humano, relativamente ao conteúdo de ADN, imunoexpressão da proteína p53 e taxa de proliferação avaliada pela imunoexpressão da proteína Ki-67

- Avaliar o significado biológico da presença de lesões de CIS na mucosa urotelial, no sentido de, no futuro, estudar se esta lesão é biológica e patologicamente equivalente no modelo animal.

MATERIAL E MÉTODOS

Modelo Humano

Nesta série foram incluídos doentes com CUB diagnosticados e tratados no IPOPFG – EPE, entre 1989 e 1996, para os quais existia consentimento informado e material de arquivo, incluído em parafina, suficiente para os estudos de imunohistoquímica e citometria de imagem. Os doentes foram tratados por RTU ou CR, consoante o seu estádio clínico, e nenhum foi submetido a qualquer tratamento prévio de radioterapia ou quimioterapia neoadjuvante.

Modelo Animal

Neste modelo animal foram utilizados murganhos da estirpe C3H/HE com 5 semanas de idade, provenientes do Instituto Gulbenkian de Ciência (Oeiras, Portugal) e ratas Fisher 344 com 5 semana oriundas de Harlan (Amesterdão, Holanda).

Os protocolos de aclimatização e alimentação dos animais, e protocolo experimental foram aprovados previamente pelo Comité de Ética Português para a Experimentação Animal (Direcção Geral de Veterinária, despacho nº 520/000/000/2003).

O agente carcinogénio utilizado para indução dos tumores na bexiga foi a BBN, adquirida à Tokyo Kasey Kogyo (Tóquio, Japão) e administrada na água para beber, numa concentração de 0,05%.

Todas as amostras histológicas de ambos os modelos foram revistas por patologistas especialistas na área (T.A. no modelo humano e C.L. e P.O. no modelo animal). Para todos os casos, o estadiamento patológico foi definido de acordo com o sistema do American Joint Committee on Cancer [Greene, 2002] e o tipo e grau histológico segundo os critérios de 1998 ou 2004 da World Health Organization [Lopez-Beltran, 2004; Sauter, 2004], consoante o indicado na altura da realização do estudo.

Avaliação do Conteúdo de ADN

O conteúdo de ADN celular foi avaliado pelo citómetro de imagem CAS 200 (Cell Analysis System, Inc. Elmhurst, Ill). Os protocolos de marcação, aquisição e análise dos histogramas de ADN encontram-se descritos na literatura [Oliveira, 2005; Santos, 2003b]. Resumidamente, cortes histológicos de 6 µm desparafinados foram corados pela técnica de Feulgen através do kit de marcação para ADN (Cell Analysis System, Inc.). Após a calibração do citómetro de imagem, foram lidos em cada corte histológico 20 a 30 linfócitos (população controlo ou de referência diplóide) e um mínimo de 100 células de interesse, intactas, não sobrepostas. Em cada histograma de ADN, foram identificados os picos G0/G1 da população controlo e da população de interesse e calculadas a média, o desvio padrão e o coeficiente de variação. Para todos os casos foi calculado o Índice de ADN, variável que descreve numericamente o conteúdo de ADN relativo da população em estudo, através da divisão entre a média do pico G0/G1 da população de interesse e a média do pico G0/G1 da população controlo. A percentagem de células com conteúdo de ADN acima de 5n (5cER), tidas como células verdadeiramente aneuplóides, foi igualmente calculada em todos os casos.

Análise Imunohistoquímica

Amostras de humanos: Nesta técnica foi utilizado o método da avidina-biotina e peroxidase, descrito na literatura [Santos, 2003a]. A expressão da p53 foi avaliada com a marcação pelos anticorpos primários DO7 (diluição 1:50, Dako) e PAb-240 (diluição 1:50, NeoMarkers - LabVision), e a expressão da Ki-67 pelo anticorpo primário MIB1 (diluição 1:50, Novocastra).

Amostras de roedores: Nesta análise foi empregue o método da estreptavidina-biotina e peroxidase, descrito na literatura [Oliveira, 2006b]. Para a expressão da p53 foi utilizado o anticorpo primário AB-1 (diluição 1:50, NeoMarkers - LabVision) e para a Ki-67 o anticorpo anti-Ki-67 (1:20, Dako).

Todos os cortes histológicos foram reavaliados por dois observadores independentes (T.A. e L.S. para a série de humanos e P.O. e C.L. para a de murinos), sem conhecimento prévio dos dados clínicos. O método de avaliação

utilizado encontra-se igualmente descrito nos respectivos artigos. A percentagem de células positivas (num mínimo de 100 células analisadas) para cada um dos marcadores imunohistoquímicos foi calculada na área de maior marcação da lesão em estudo. Na série humana os valores "cut-off" utilizados foram >18% e >32%, para a p53 e Ki-67, respectivamente; na série de roedores a imunoexpressão dos dois marcadores foi calculada pela divisão entre a percentagem de núcleos positivos e o total de núcleos analisados.

Análise estatística

A análise estatística foi realizada, nos vários estudos, recorrendo ao programa estatístico SPSS (SPSS, Inc., Chicago, IL), tendo-se, inicialmente, procedido a uma análise estatística descritiva de todas as variáveis em estudo. Em cada artigo foram utilizados os testes estatísticos adequados ao tipo de variáveis e, em cada um dos testes, as diferenças foram consideradas estatisticamente significativas quando $p<0,05$.

ESQUEMA DA TESE

MODELO HUMANO

Artigo #1: DNA image cytometry in bladder cancer: State of the art.

Palmeira CA, Oliveira PA, Seixas F, Pires MA, Lopes C, Santos L.

Anticancer Res. 2008; 28: 443-450.

ANTICANCER RESEARCH *28*: 443-450 (2008)

Review

DNA Image Cytometry in Bladder Cancer: State of the Art

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Abstract. *The most recent Consensus Review of the Clinical Utility of DNA Cytometry in Bladder Cancer, which took place in Maine back in 1992, focused solely on flow cytometry results. Since then, there have been a significant number of articles published on the use of image cytometry to evaluate DNA content in bladder cancer. This has meant that a large proportion of the information collected is scattered across the published literature. The purpose of this article was to organize the data referred to in those articles published since the 1992 Consensus Review, and organise it under three major topic headings: a) DNA image cytometry versus flow cytometry, (b) specimen sources, and (c) its clinical utility with regard to improving prognosis and recurrence detection. A variety of factors and issues are discussed and points have been raised for discussion. Prospects for the future and potential research areas are also suggested.*

In 1992, a DNA Cytometry Consensus Conference (1) was held in order to evaluate the clinical applications and technical aspects of DNA ploidy analysis in patients with transitional cell carcinoma of the bladder (TCC). A highly significant review of the most representative results published on this issue took place at this gathering, which led to the establishment of a series of consensus conclusions and guidelines. However, flow cytometry (FCM) analyses were the only form of analysis considered, the reasoning behind this decision being a perceived paucity of reports on image analysis in bladder cancer.

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Key Words: DNA content, image cytometry, bladder cancer, review.

A number of guidelines were laid down with regard to the application of FCM in the DNA content analysis of bladder samples, specimen sources, preparation and staining, and data analysis. Various technical factors, such as optimal staining protocols, biological specimen and key factors of histogram interpretation were also discussed.

Following the recommendations that resulted from this Consensus Conference, the last fifteen years have seen the appearance of several published articles on the use of image cytometry (ICM) to evaluate DNA content in bladder cancer in a bid to define its usefulness from a clinical and prognostic perspective. Some studies compared ICM with FCM, while others examined its association with other biological markers. The purpose of this review was to help to organize this widely scattered collection of data and to discuss certain aspects of the correlations discovered and the prognostic value identified in the distinct studies, and then compare these findings with our own experience.

DNA Image Cytometry vs. Flow Cytometry

The DNA content of neoplastic cells is generally evaluated using FCM, though quantitative microscopic image analysis is a reliable alternative technique for quantifying of DNA content. Both methods have been used to measure the extent of genetic alteration in bladder lesions. Despite the positive overall correlation that the published literature draws between these two methods particularly when it comes to cytological specimens (2, 3), they each have their own particular advantages.

FCM is quicker to perform than ICM, enabling a greater number of cells to be measured and therefore resulting in a lower coefficient of variation (CV). Offsetting these positive features, the sensitivity of FCM analysis may suffer from a lack of necessary visual discrimination to enable non-tumour

0250-7005/2008 $2.00+.40

cells and artefacts to be excluded from the measurements. At the aforementioned Consensus Conference (1), the need to enrich the proportion of tumour cells present in order to achieve an adequate level of sensitivity when using FCM was highlighted, especially with paraffin-embedded material where section trimming is sometimes required. The relatively small number of cells in each sample can also make the FCM analysis less reliable (4-6).

These issues can be avoided with ICM, by microscopically selecting only the tumour cells. ICM's higher sensitivity when used on small populations of aneuploid cells is noted by several authors (2, 3, 5-13), particularly in tetraploid cell clones (9, 10). Nevertheless, this relatively high resolution can wane in those histograms with near-diploid cell populations, which due to this technique's higher CV cannot be accurately classified as true near-diploid or right shifted diploid populations (2, 3, 6).

The need for ICM to be performed by skilled operators and the fact that it is a very time-consuming method are both significant limitations and combine to reduce its cost-effectiveness (6, 14). Moreover, the interactive operator-guided selection of the nuclei can cause selection bias and low reproducibility (6). Some authors claim that these disadvantages can be offset by an automated image cytometry system (6, 15). Baak *et al.* (6) proposed a highly automated system, developed in their own laboratory, based on an automated motorized microscope and scanning platform. Measurement efficiency is improved using densitometric and geometric filters based on quantitative cell features, such as area, integrated optical density, membrane roundness, convex perimeter and curve width. That said, these authors did mention that the system had some weaknesses, given that single-cell preparation is required and the resulting histograms can sometimes be inadequate. According to the authors, these kinds of automated systems can bring CV values and the time required for each measurement very close to those obtained using FCM analysis. In our opinion, the lower CV value and time required are of course important advantages of these more automated systems. However, operator experience is one of the most important features of ICM and should not be restricted by automatic analysis. This experience is vital for distinguishing between certain neoplasic and preneoplastic lesions, whether pathological or reactive cells, and offers a high level of sensitivity when it comes to detecting small aneuploid peaks. In future, image analysis systems should take these two differing points of view into consideration.

DNA Image Cytometry and Specimen Sources

According to the guidelines set out at the 1992 Consensus Conference, voided urine was not suitable for DNA FCM analysis due to poor cell yield and the cellular aggregation often presented in these samples (1).

The specific features of ICM, as mentioned in the previous section, make it a suitable method for studying cytological samples such as voided urine and bladder washings. Nevertheless, certain samples should be analysed with caution. A case in point are the nuclei of superficial (umbrella) cells that often have abnormal DNA content, which may cause abnormal DNA ploidy results in cytomorphologically normal bladder washes. Umbrella cells should be avoided in the evaluation of urine samples (16).

In the study carried out by Forte *et al.* (17), the DNA ICM results obtained from different specimen sources: cytologic smears, cytocentrifuge samples, tissue sections (5-micrometer sections) and nuclei extracted from paraffin-embedded tissue were compared. Using a binary DNA classification scheme (diploid or aneuploid), they found comparable DNA content results across the different samples: bladder washings and tissue section results coincided in 40 out of 43 patients.

Mainguene *et al.* (18) observed differing results when they compared touch imprints with formalin-fixed paraffin-embedded samples. They made imprints of fresh specimens from 31 urothelial carcinomas biopsies and cell suspensions were obtained from dewaxed tissue sections (7 microns) using the Hedley procedure (18). These authors stated that imprints were a simple and reliable procedure for DNA ploidy assessment which were particularly sensitive when it came to detecting small aneuploid peaks and multiploid tumours.

As far as our experience with a variety of biological tissues are concerned (*e.g.* breast, bladder and lymph nodes), cytological preparations, especially from fresh material, provide highly suitable source material for DNA ICM evaluation, with CV values near those obtained by FCM. However, in those studies where different compartments of the tissue must be evaluated, namely where the adjacent mucosa needs to be evaluated, then tissue sections must be used. However, there are certain technical problems that must be considered when using these tissue sections. These include: section thickness, sliced nuclei resulting in a low DNA ploidy estimate, overlapping nuclei that result in an overestimation of nuclear DNA content, and the time-consuming analysis that requires many nuclei be sliced apart or rejected (19). We believe that the 6-Bm section thickness used in our laboratory is a suitable compromise between the need for minimal nuclear overlap and sharp focusing, and the need for full nuclear thickness. Given the DNA content estimates made from sliced nuclei, internal reference cells should be used rather than external populations. These internal reference cells may be infiltrated lymphocytes or granulocytes present in the tissue, or normal epithelial and stroma cells as stained in the ESACP DNA Consensus in Image Cytometry (20). In our own experience, infiltrated

lymphocytes are a suitable reference cell population. When cytological specimens are used and external reference cells are required cells such as rat liver hepatocytes or lymphocytes, from the same animal species as the pathological cells, can be used. In a previous study using normal mice we evaluated imprints from a variety of sources (spleen, lymph nodes and thymus). We found that spleen samples were the material best suited to DNA content evaluation, due to the higher number of intact and non-overlapping lymphocytes and better-stained samples (21). These external reference cells should be prepared and fixed in the exact same way as the cells under analysis.

DNA Image Cytometry and Clinical Utility

Screening. One of the conclusions drawn by the Consensus Conference was that DNA FCM should not be used for screening for bladder cancer nor for investigation of microscopic hematuria. In spite of its higher sensitivity for detecting bladder cancer, in comparison with urine cytology, its specificity remains too low especially in low-risk patients (1). In the years that followed this Consensus a number of articles have evaluated the clinical utility of DNA ICM analysis, compared with conventional urinary cytology (11, 22-28) or with others methods such as fluorescence *in situ* hybridization (FISH) (5, 11, 29, 30), or immunocytology (24). In general terms, the authors agree that DNA ICM has a higher level of sensitivity than cytology, particularly in grade 1 tumours. Planz *et al.* (23) studied spontaneously voided urine from patients with histologically proven TCC and patients with benign bladder diseases and found that DNA ICM was significantly more sensitivity for detecting grade 1 tumours (70.4%) than cytology (26%).

Despite these results, Shabaik *et al.* (22) alert readers to the fact that some low-grade lesions with DNA diploidy and a low proliferation index can pass undetected by ICM. Furthermore, Katz *et al.* (31) point out that in patients with no history of TCC, abnormal DNA ICM patterns can occur after chemotherapy, radiation therapy, or viral infection. They suggest that a combined approach using both cytology and DNA ICM, alongside an accurate record of the clinical history is essential for correct diagnosis.

DNA ICM sensitivity can be increased with the use of other stemline analysis systems, when compared with the conventional classification based on the 2c deviation index (23). Some authors suggested the stemline interpretation system presented by Böcking *et al.* (32) which used the Kolmogoroff-Smirnow test (23, 33, 34), while others developed their own analysis system (27).

An image analysis system described in the literature and referred to as a test for bladder tumours is the Quanticyt Karyometric System. This automatic image analysis system evaluates the nuclear shape and DNA content of exfoliated cells in bladder wash specimens to detect bladder cancer and/or identify individuals at low or high risk for bladder cancer development (13). According to Lokeshwar *et al.* (25) this system may overestimate the risk of urothelial abnormalities and, therefore, have a lower specificity than bladder wash and urine cytology. Furthermore, the low number of urothelial cells or an abundance of leucocytes and erythrocytes present in the sample may limit its use.

Urine cytology is still very widely used for diagnosing bladder cancer, in spite of its limited sensitivity and subjective nature, due to its high specificity. Therefore, cytology could be used in combination with DNA ICM and others methods such as FISH to improve overall sensitivity levels without affecting the specificity. More studies ought to be carried out to evaluate this combined method given the relatively expensive and time consuming nature of ICM and FISH, prior to these methods being established as clinically routine.

Prognosis. Transitional cell carcinoma of the bladder is a tumour with varying biological potential and, therefore, with a wide spectrum of risks of recurrence or progression. The recurrence rate ranges between 50% to 70% and as many as 19% of pTa and 34% of pT1 tumours will progress into muscle-invasive tumours (14). With regard to this group of superficial TCC (sTCC), excluding the high-risk tumours (T1/G3 and *in situ* tumours), there is no established consensus treatment, and the classical histopathological markers do not define risk profiles. Consequently, there are no optimal treatment approaches or follow-up schedules (35). Several biological variables have been studied in order to try and obtain additional information that would facilitate patient treatment selection and improve patient survival.

Based on a review of the available literature and in reference to sTCC (Ta, T1 TIS), the Consensus Conference stated that DNA FCM correlates with grade and stage, and provides prognostic information regarding stage progression, muscle invasion and metastasis. The authors stated that the DNA ploidy status listed in order of decreasing risk of progression was: multiple DNA aneuploid populations > single DNA aneuploid populations > DNA "tetraploid" population > DNA diploid populations (1). With regard to muscle-invasive tumours (T2-T4), the Consensus concluded that the data concerning the role of DNA FCM was inconclusive and therefore did not provide a sound basis for treatment recommendations (1).

Over the last fifteen years, several studies have evaluated the prognostic value of DNA ICM in sTCC. A variety of cytometric parameters have been singled out as being useful in predicting survival and progression-free survival in this group of bladder cancer patients: DNA ploidy (6, 14, 36-43), mean ploidy (44), 2cDI (8, 44-46), proliferation index (47), DNA index (6), percentage of hyperdiploid and hypertetraploid cell

nuclei (48), and 5cER or percentage of polyploid cells (36, 46, 49). Some authors wrote that this prognostic value is particularly important in grade 2 tumours (17, 48, 50). This group of tumours is very heterogeneous in respect of their clinical behaviour and DNA ploidy, although they exhibit the same degree of histomorphological differentiation. According to al-Abadi and Nagel (50), these tumours can be subclassified as aneuploid (biologically aggressive), diploid or tetraploid (biologically less aggressive). Decaestecker *et al.* (48) evaluated nine different ploidy-related parameters *via* a computer-assisted method, selecting two of them (percentage of hyperdiploid and hypertetraploid cell nuclei) which enabled the identification of two subgroups within grade 2 tumours: ones which exhibit clinical behaviour similar to grade 1 tumours and a second subgroup which exhibit clinical behaviour similar to grade 3 tumours.

Our group, based on the findings of experimental studies on laboratory animals (rats and mice), also reported this discriminatory potential between lesions with the same degree of histomorphological differentiation showed by DNA ICM: the same histological lesion revealed differing aneuploidy frequencies depending on the length of exposure to *N*-butyl-(*N*-4-hydroxybutyl) nitrosamine (BBN) (51-54).

From a review of the literature, it is possible to conclude that the prognostic value of DNA ICM variables in sTCC could be significantly enhanced when associated with others biological parameters, such as morphometric features (13, 37, 38, 42, 43, 45, 55). This advantageous possibility is well-described in studies by van Velthoven's group (37, 38, 42). They used different morphonuclear parameters evaluated by digital cell image analysis in relation to the chromatin pattern. When the information contributed by this quantitative chromatin pattern description was added to DNA ploidy level, histological grading and clinical staging, they obtained a higher prediction value for biological behaviour in sTCC (38).

Another biological marker that has been studied alongside the DNA content status of the sTCC is the proliferation rate. Different proliferation markers have been evaluated such as proliferating cell nuclear antigen expression (39, 40, 46, 56), Ki-67 labelling index (52, 57-60) and mitotic index (58). In general, all these studies refer to an improvement in the predictive value of cytometric features with respect to the potential malignancy of sTCC when measured simultaneously with proliferation markers. They also state that recurring cases are less frequently found in the group of DNA euploid tumours exhibiting a low proliferation rate, in contrast with a very high percentage of recurrence in patients with DNA aneuploid tumours with a high proliferation rate.

As regards muscle-invasive TCC, the prognostic value of DNA ICM is as controversial as DNA content evaluation by FCM. Cai *et al.* (14), based on 10 years of follow-up examinations after surgery on 8 invasive bladder tumours, found no significant difference between patients with a normal DNA content and those with aneuploid DNA content. In contrast, Decaestecker *et al.* (61) state that nuclear DNA content and chromatin pattern-related parameters can provide prognostic information for this TCC group. These authors studied 41 patients and, using a number of image-generated variables produced *via* a computer-assisted methodology, they were able to characterise the tumours associated with a "bad" prognosis in the T2 sub-group and the tumours associated with a "good" prognosis in the T3-T4 one.

These conflicting results in the invasive TCC may be partially related to the kind of patients included in each study. Rotterud *et al.* (62). evaluating 94 patients treated with external pelvic radiotherapy, found a better rate of survival in the aneuploid tumours group compared with their diploid tumours counterparts. According to these authors, this discovery could be due to the increased radiosensivity of aneuploid cells, as demonstrated by others (63-65). More studies with comparable patient groups and treatment schemes are required in order to clarify the prognostic potential of DNA ICM in invasive TCC.

Detection of recurrence (follow-up). Cystoscopy remains the gold standard in the detection of TCC recurrences. Other methods have been studied in an attempt to reduce the frequency of follow-up cystoscopies in patients with a low risk of tumour recurrence, and thus reduce costs and patients discomfort, and can help in the stratification of treatment modalities (13).

For patient follow-up, the Consensus Conference established that taking a bladder irrigation specimen was their preferred technique of sampling the urothelium in DNA FCM analysis. Of the differing cytometric variables related to DNA content, a clear "nontetraploid" DNA aneuploid histogram proved to be the most informative parameter, as far as tumour recurrence and treatment failure were concerned (1). A DNA diploid histogram, however, does not exclude tumour recurrence and should be evaluated carefully (1).

With regard to DNA ICM, a variety of urine sample types have been studied and shown to be suitable for evaluating the DNA content of urothelial cells, such as voided and catheterized urine and bladder irrigation (5, 10, 12, 28, 29, 31, 66-69). As previously mentioned, this is related to the ability of ICM to measure fewer cells in smaller samples and its higher sensitivity for detecting aneuploid cells at extremely low frequency levels, when compared with FCM.

As previously mentioned, a combination of visual cytology of urine specimens with DNA ICM offers a better method for the early detection of recurrent TCC, than cytology alone (5, 12, 29, 31, 66, 67, 70, 71). In the literature, sensitivity

values of between 76-93% were reported as well as a specificity of between 41-94% (12). Different patient selection methods and a different histogram analysis scheme may explain this variation (various cytometric parameters have all been shown to be informative: DNA ploidy, 2cDI, hyperdiploid fraction and 5cER).

Authors are in agreement on one issue, an abnormal DNA content (nontetraploid histogram) is 100% specific for tumour recurrence. For negative cytology and normal DNA content cases, this value is remarkably lower. More sensitive diagnostic tests such as chromosomal abnormalities by *in situ* hybridization are suggested for use in these cases (5, 31, 72). A case in point is the multitarget FISH probe UroVysion, which includes probes for the centromeres of chromosomes 3, 7 and 17, and for the p16 gene at chromosome 9p21 (5). Nevertheless, there are interpretative challenges involved with this kit that are caused by signal overlaps in highly aneuploid samples, while focal plane distortions and high cost place limits on its clinical applications (30).

According to Richman *et al.* (12), when the detection capacity of cytology and DNA ICM are used in combination with each other, their detection capacity is greatest when urinary samples are obtained as close as possible to the moment the disease becomes clinical by evident. The authors stated that the combination can be of predictive value up to three months prior to clinical diagnosis. In this way, patients can be treated earlier and more conservatively.

The literature also made reference to another issue that can influence diagnosis accuracy. This issue is related to the DNA tetraploid tumours, the aneuploid population most frequently missed by FCM. Kline *et al.* (10) found in their study that 45% of cases, within this tetraploid group, exhibit no clinical (cystoscopic) or pathological (cytologic and histologic) evidence of neoplasia, and none of these patients developed tumours during follow-up. The authors suggested that the presence of DNA tetraploidy in cytologically negative cases should be interpreted cautiously. These findings are in agreement with the known occurring physiological polyploidy of urothelial cells. According to Planz *et al.* (23), for example, 4c and 8c ploidies could be detected in 90% of normal urothelial tissue samples. The use of stemline interpretation instead of single-cell interpretation could be used to overcome difficulties in discriminating between physiological polyploidy and the real abnormal tetraploid DNA content. The DNA stemline is defined as the most frequently occurring c value in a tumour cell population, if accompanied by cells at its twofold DNA value (23).

From literature reviewed in this work, it is possible to conclude that DNA content evaluation using ICM provides prognostic information in TCC, especially if other biological parameters, such as morphometric features and proliferation markers, are studied alongside it. This clinical value is not so evident when used in screening and recurrence detection due to the limited specificity of DNA ploidy. However, when combined with visual cytology, it offers a better method of bladder cancer detection than cytology alone.

In spite of the clinical utility showed by the DNA ICM, the heterogeneity between the different DNA parameters and the DNA histogram interpretation system included in each study contributes to some variation in the inter-laboratory reproducibility of the DNA content results. Moreover, the routine clinical use of current image analysis systems is limited because it remains a very time-consuming method and is dependent on the operator's skill level. The more automated image cytometry systems that have been developed and referred to above may overcome some of these drawbacks. However, further improvements are required to reduce the costly and elaborate preparatory procedures needed to produce cellular specimens suitable for automated analysis. These new systems must also consider operators as factors for potential improvement as opposed to limiting variables. This is because operator experience is the single biggest contributor to ICM's sensitivity and discriminatory potential.

DNA ploidy alterations are a global marker of genomic instability and chromosomal derangement. ICM is a suitable method of evaluating this genetic instability in distinct areas of a tissue section. This feature could prove to be a powerful tool in the study of biological mechanisms in the carcinogenesis process.

There is a great deal of published literature related to the study of DNA content in TCC. Most of this was focused on clinical and prognostic correlations rather than the biological mechanisms and possible role of DNA ploidy changes in the therapeutic response level. Following on from these areas of research, our group has published some data that could identify subgroups of urothelial cell carcinomas that correlate with a poor response to current therapies (surgical and chemotherapy approaches) and a less favourable clinical prognosis. This is the case with DNA diploid tumours induced by BBN in rats which were less sensitive to mitomycin C and bacillus Calmette-Guérin instillation (52), and a subgroup of invasive tumours in a series of human TCC that exhibited a decrease in proliferation rate, as measured by Ki-67 immunohistochemistry, despite their more frequently occurring DNA aneuploid pattern (60). The latter group of tumours was confirmed by the results found by Grossman *et al.* (73). In a series of locally advanced bladder cancer that were randomly treated using either neoadjuvant chemotherapy plus cystectomy or cystectomy alone, the results showed a less favourable progression-free survival rate for those patients whose tumours exhibited a lower expression of Ki-67, although it was only marginally significant.

As regards the therapy schemes now available for superficial and invasive TCC, it will be interesting to discover what the distribution of the DNA content pattern really means and if this biological information could help us to better understand and identify those cases which respond poorly to treatment. The issue lies in discovering if DNA ploidy changes could be a critical variable in designing a bladder nomogram, which would help physicians to decide which treatment method will be of the greatest benefit to their patients. For those tumours that do not respond to treatment, other therapeutic targets must be investigated in due course in order to lay down alternative treatment approaches.

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Received October 30, 2007
Revised December 18, 2007
Accepted December 24, 2007

Artigo #2: Is DNA content alteration a consequence of proliferative and differentiation changens in urothelial bladder tumours?

Santos L, Lameiras C, Afonso J, Palmeira C, Pereira S, Costa C, Amaro T, Bento MJ, Morais A, Criado B, Lopes C.

Acta Urol 2003; 20: 9-17.

Acta Urológica 2003, 20; 1: 9-17

Is DNA content alteration a consequence of proliferative and differentiation changes in urothelial bladder tumours?

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Abstract

The loss of differentiation, high cell proliferation rate and aneuploidy are usually related with tumour aggressiveness. The accumulation of this phenotype in bladder cancer was observed in invasive tumours. However in superficial ones it is not clarified the meaning and the time of these events. We studied simultaneously cell proliferation, blood group ABH antigen expression and DNA ploidy in urothelial cell bladder carcinoma in order to determine the relationship between these biomarkers and clinical-pathological features of urothelial cell carcinoma of bladder.

In present series we verified that early tumours (Ta/G1) had high proliferative index, loss of blood group antigen expression and were DNA diploid. Invasive tumours (T2/T4G3), also showed loss of A, B and H antigens expression, but were more frequently DNA aneuploids and showed a decreased in proliferation rate.

These results suggest that aneuploidization, i.e., DNA content alteration, might be a consequence of these previous changes.

Resumo

A perda de diferenciação, um elevado índice proliferativo e a aneuploidia são características biopatológicas associadas a maior agressividade tumoral. Este fenótipo é observado com frequência nos tumores invasores. No entanto, nos carcinomas superficiais da bexiga, o significado destes eventos e o momento em que ocorrem não estão esclarecidos.

Estudámos simultaneamente a proliferação celular, a expressão dos grupos sanguíneos e a ploidia de DNA, numa amostra de carcinomas uroteliais da bexiga, com o objectivo de determinar a relação entre estes marcadores biológicos e as características clínico-patológicas.
No presente estudo, verificámos que os tumores mais precoces (TaG1) tinham um índice proliferativo elevado e perda de expressão dos grupos sanguíneos, sendo, no entanto, diplóides de DNA. Os tumores invasores (T2/T4G3) tinham perda de expressão dos antigénios ABH; eram, na sua maioria, aneuplóides de DNA, e a taxa de proliferação era baixa.
Estes resultados sugerem que o processo de aneuploidização, entendido como alterações do conteúdo de DNA, é o resultado da perda de diferenciação e de uma elevada taxa de proliferação.

Introduction

DNA aneuploidy, which indicates a state with an abnormal DNA and chromosome content, has been found in various human cancers[1]. This parameter is considered to be an important biological and prognostic variable in human cancer[2-3].

In bladder carcinoma some authors have reported an objective assessment of grading by cytophotometry, achieving a good correlation between the histological grade of bladder tumours and the DNA content[4]. This correlation was well reported in the Consensus on the clinical utility of DNA ploidy analysis in patients with transitional cell carcinoma of the bladder[5]. Wheeless et al[6] found that the majority of grade 1 bladder tumours were DNA diploid, being the majority of grade 3 tumours DNA aneuploid. Furthermore, superficial bladder cancer, compared with the invasive ones, had a significantly lower number of aneuploid cells[6].

Image cytometry studies report that it is possible to determine the ploidy level in a given tumour using the parameters obtained from the DNA histogram, assessed by Feulgen-stained nuclei. One of them is the DNA histogram type (DHT), whose parameters contribute with discriminatory information on the clinical progression in superficial transitional cell carcinomas of the bladder[8]. The percentage of aneuploid cells with DNA content above 5c (5cER) is another ploidy related parameter, and is important in assessing bladder cancer aggressiveness, being a reliable biomarker in the risk assessment for this type of tumour[8-9].

The histoblood group ABO involves three carbohydrate antigens - A, B, and H. A and B and AB individuals express glycosyltransferases that add sugar residues to H structure, whereas O individuals lack such activity [10]. The ABH gene is mapped on 9q34.1--34.2 region [11]. The presence of blood group antigens (A, B, H) on the surface of the epithelial cells depends on the individual secretor status. About 85% of the population is secretor and express A, B, and H in their normal urothelium[12]. Alterations of the blood group antigen and loss of ABH gene chromosomal region are frequent in bladder cancer. This antigen loss has been reported in bladder cancer as related to progression and poorer prognosis[13-14]. De Cenzo et al [15] and Newman AJ et al[16] observed, particularly among superficial papillary urothelial carcinomas, progression in those with blood group antigens (A, B, H) loss.

Proliferation rate assessed by Ki-67 labelling index (LI) was recently accepted as a prognostic marker in superficial papillary urothelial carcinomas of the bladder[17]. Tumours with high LI show a significant low recurrence-free and progression-free survival[17]. Bush et al [18] observed that invasive bladder tumours had a significantly higher Ki-67 LI than the non-invasive ones. Also, bladder tumours with lymph node involvement had higher LI than those without lymph node metastasis, appearing that the high frequency of S-phase cells within the tumour tissue to indicate a great potential for malignancy and a poor prognosis[19].

The prognostic value of blood group ABH antigen status and DNA ploidy combination, assessed by flow cytometry, was related to the disease outcome [20]. On the other hand, more than 10% of cells with 5cER of DNA content were related to the increase of the cell proliferation rate[21]. However, the relationship between grade and stage and the combination of these three biological markers (cell proliferation, blood group ABH antigen expression and DNA ploidy) has not been performed simultaneously, until now. Therefore, the aim of this study was to analyse the

usefulness of image DNA ploidy and other ploidy--related parameters, blood group antigens (ABH) status and Ki-67 labelling index in predicting prognosis and for therapeutic purposes.

Patients and Methods

This study comprised 94 unselected patients diagnosed between 1990 and 1996 at the Instituto Português de Oncologia, Porto, Portugal; 53 with primary superficial (56.4%) and 41 invasive (43.6%) urothelial bladder carcinoma. The patients' age ranged from 28 to 83 years with a median age 73 years. The patients were treated either by transurethral resection or cystectomy; no previous radiation or chemotherapeutic treatments were performed. All specimens were graded using the World Health Organization (WHO) classification[22] and staged according to the AJCC system[23]. In the series, 68 (72.3%) tumours were papillary, 19 (20.2%) were non-papillary and the 7 (7.5%) remaining were mixed. Ten (10.6%) cases were G1, 50 (53.2%) were G2 and the last 34 (36.2%) cases were G3. The stage of tumours was Ta in 9 (9.6%), T1 in 44 (6.8%) and T2-T4 in 41 (43.6%).

Image Analysis

The nuclear DNA content of cancer cells was measured with CAS 200 image analysis system (Cell Analysis Systems, Inc., Elmhurst, Ill.). Sections from paraffin-embedded blocks were cut at 6 μm and were deparaffinized and rehydrated. The slides were stained by standard Feulgen method with the DNA staining kit (Cell Analysis System, Inc.), according to the manufacturer's instructions. Within each staining batch was also stained one CAS slide with control rat hepatocytes. The image system was first calibrated using the controls, which contained a known quantity of DNA. Twenty to 30 lymphocytes and a minimum of 100 intact non-overlapping tumour nuclei were measured and analysed for each case, using the Quantitative DNA Analysis software program (Cell Analysis System, Inc.). The integrated optical density (OD) of each Feulgen-stained nuclei was considered proportional to the amount of DNA present in the nuclei. The OD of lymphocyte nuclei from each section served as internal control (diploid reference). The resultant DNA histograms were analysed by previously used methods [24]. For each tumour G0/G1 peak visually identified, mean, standard deviation (SD) and coefficient of variation (CV) values were calculated. The control CV provides an indicator of overall precision of the imaging technique. The DNA index (DI) was evaluated as the ratio of tumour G0/G1 peak mean value divided by the internal control lymphocytes G0/G1 peak mean value. Peaks having a DI greater than 3SD from the internal control lymphocytes were considered aneuploid. The 5cER was also evaluated being defined as the percentage of tumour nuclei with DNA values above 5n.

DHT – DNA Histogram Type

To obtain a reproducible DNA histogram classification, the DHT were classified into five categories (Table 1).

Type 1 - a single distinct G0/G1 peak with a DI within 3SD from the internal control lymphocyte (diploid region) containing at least 70 % of its cell nuclei populations[8], usually with less than 1 % of 5cER

Type 2 - a single G0/G1 peak in the near-diploid region or two G0/G1 peaks, one in the diploid region and the second peak in the near-diploid region

Type 3 - show either a distinct modal value in the tetraploid or near tetraploid region or have two well-defined peaks around the 2n and 4n regions

Type 4 - have a single DNA aneuploid peak outside the tetraploid or near-tetraploid region

Type 5 - contain more than two distinct G0/G1 peaks showing a very pronounced and irregular aneuploidy, with DNA amounts ranging from 2n up to values exceeding 6n or even 9n

Table 1 – Definition of DHTs identified in bladder carcinomas analysed by Image Cytometry

DHT Type	DI Range Value of Tumour G0/G1 DNA Peak
1	$0.83 < DI \leq 1.17$
2	$1.17 < DI \leq 1.20$
3	$1.90 < DI \leq 2.20$
4	$1.20 < DI \leq 1.90$ and $DI > 2.20$
5	—

Table 2 – Studied biomarkers in function of stage and grade

	n	*5cER*		*DHT*					*DNA ploidy*		*Ki-67*			*Blood group ABH antigen*			
		without	with	1	2	3	4	5	diploid	aneuploid	neg	1-18%	≥18%	loss	partial loss	expression	NS
TaG1	5	5	0	5	0	0	0	0	5	0	0	2	3	3	2	0	0
T1G1	5	3	2	2	1	0	2	0	2	3	2	2	1	2	0	2	1
TaG2	4	2	2	2	0	0	0	2	2	2	1	1	2	1	2	0	1
T1G2	33	13	20	6	5	0	9	13	6	27	11	11	11	14	9	8	2
T2-T4G2	13	3	10	3	0	1	2	7	3	10	5	2	6	5	4	1	3
G3	34	1	33	3	1	3	4	23	3	31	25	5	4	27	4	3	0
Total	94	27	67	21	7	4	17	45	21	73	44	23	27	52	21	14	7

5cER - 5c exceeding rate. DHT - DNA histogram type. NS - non secretor. Neg - negative

Immunohistochemistry

Sections of 4μm were cut from formalin-fixed, paraffin-embedded primary bladder cancers. A microwave oven was used for antigen extraction; endogenous peroxidases were blocked by incubation with 0.3% hydrogen peroxide. The slides were incubated with normal horse serum (VectaStain ABC kit, Vector Laboratories®) for 20 min at room temperature. Tissue sections were then incubated with primary antibodies. Monoclonal antibodies anti-blood group antigen A (1/100 Dako®), antigen B (1/15 Dako®) and antigen H (1/15 Dako®) were incubated for 12 minutes at room temperature. Mib-1 (1/50 Novocastra®) was applied overnight at 4ºC. These sections were sequentially incubated with the secondary biotinylated antibody (VectaStain ABC kit, Vector Laboratories®) and avidin-biotin peroxidase complexes (VectaStain ABC kit, Vector Laboratories®) for 30 min. Reaction products were revealed with diaminobenzidine (DAB) as the chromogen. The sections were counterstained with Harris's hematoxylin.

Paraffin-embedded tissues known to express nuclear Ki-67 in more than 18% of the cells (lymphoma) were used for titration and positive controls. Negative controls were performed by replacing the primary antibody for 2.5% bovine serum albumine (BSA) in phosphate-buffered saline (PBS). For A, B and H immunoreactivity, tissues expressing the appropriate blood group antigen were used for titration of reagents as well as for the positive and negative controls. In addition, stained endothelial and red blood cells present in the tissue sections served as internal positive controls for the secretor phenotype[25].

Immunohistochemical scoring

Positive staining was defined as the presence of ≥ 18% antibody-stained nuclei for Ki-67, according to the cut-off values of the National Cancer Institute Bladder Tumour Marker Network[26]. Secretor tumours were classified according to the semiquantification of A, B and H loss of expression in membrane and/or cytoplasm, being the three categories: total loss of expression, partial loss of expression and with expression. The entire section was screened to find the region with maximum fraction of positive Ki-67 cells and the region without cells stained for ABH blood group antigens. The percentage of positive stained nuclei was scored in this region using the 40x objective. At least 200 malignant cells were counted in each case. Immunohistochemical evaluation was done by two independent observers (T.A. and L.S.).

Statistical analysis

A descriptive study was performed for all variables included in the study. Chi-square and Fisher's exact tests were used. All statistical analyses were performed with SPSS 8.0 software (SPSS Inc.). A $p<0.05$ was accepted as statistically significant.

Results

From the 94 urothelial bladder carcinomas studied, 73 (77.7%) were DNA aneuploid and 21 (23.3%) were diploid tumours. Type III DNA histogram was the most prevalent, having 71% of the tumours 5cER cells (Table 2). The A, B and H expression was lost in 52

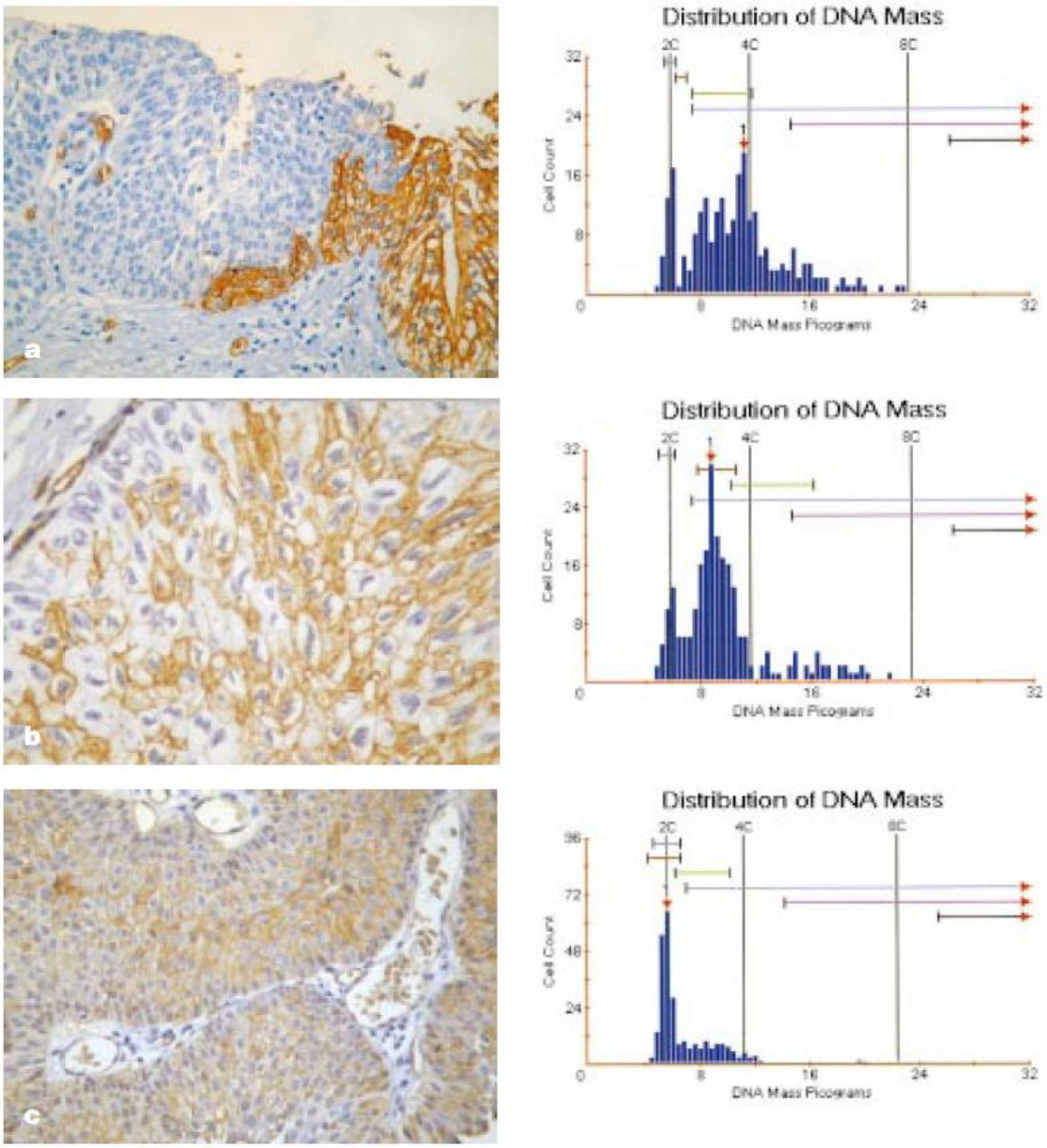

Figure 1

a) Total loss of blood group antigen (ABH) in superficial urothelial bladder cancer and corresponding DNA aneuploid histogram type V (transitional area between tumour and normal mucosa), Ki-67 LI - 0%.

b) partial loss of blood group antigen (ABH) in superficial urothelial bladder cancer and corresponding DNA aneuploid histogram type IV, Ki-67 - 5%.

c) Expression of blood group antigen (ABH) in superficial urothelial bladder cancer and corresponding DNA aneuploid histogram type I, Ki-67 LI - 17%.

(55.3%) tumours, partially lost in 21 (22.3%) and was present in 14 (14.9% - Figures 1 a, b and c, respectively); 7 (7.4%) were non-secretor cases. The immunohistochemical status of Ki-67 protein was positive (>18% of cells) in 27 (28.7%) cases, partially positive (1-18% of positive cells) in 23 (24.5%) and negative in 44 (46.8%) cases. Table 3 shows the relationship between DNA content parameters, Ki-67 protein expression and blood group antigen loss according to tumour morphology, grade and stage. Diploidy

Table 3 – Discriminatory distribution of biomarkers studied according to morphology, grade and stage

	Morphology				Grade				Stage			
	Pap	NP	M	p	G1	G2	G3	p	Ta	T1	T2-T4	p
DNA Ploidy												
Diploid	19	2	0	0.09	7	11	3	0.0001	7	8	6	0.0001
Aneuploid	49	17	7		3	39	31		2	36	35	
5cER												
Without	27	0	0	0.001	8	18	1	0,0001	7	16	4	0,0001
With	41	19	7		2	32	33		2	28	37	
DHT												
I	19	2	0	0.01	7	11	3	0.001	7	8	6	0.0001
II	7	0	0		1	5	1		0	6	1	
III	1	3	0		0	1	3		0	0	4	
IV	14	1	2		2	11	4		0	11	6	
V	26	13	5		0	21	23		1	19	24	
Ki-67												
0%	25	14	5	0.007	2	17	25	0.003	1	19	24	0.09
1-18%	18	5	0		4	14	5		3	13	7	
>18%	25	0	2		4	19	4		5	12	10	
ABH												
Expression	11	0	3	0.01	2	9	3	0.02	0	10	4	0.31
Partial loss	18	2	1		2	15	4		4	9	8	
Total loss	31	16	3		5	19	26		4	21	25	
No secretor	7	0	0		1	6	0		1	3	3	

5cER - 5c exceeding rate. ABH - blood group antigens. NS - non secretor, Pap - papillary, NP - non papillary, M - mixed. DHT – DNA histogram type.

was correlated significantly (p=0.0001) with TaG1 tumours. The percentage of cases having cells with more than 5c (5cER) was significantly correlated with G2/G3, T1 and T2-4 tumours (p=0.0001). Aneuploid histograms type (II-V) were more frequent in G2/G3 and T1, T2-4 tumours. Low proliferative LI was observed in 73.5% of G3 tumours. The majority of G2/G3 tumours lost partial or totally the ABH immunoreactivity. As can be seen in table 4, about 70% of the tumours without proliferation (Ki-67 negative cases) and 5cER cells lost totally or partially the A, B, H immunoreactivity (p=0.008).

Discussion

Molecular and cytogenetic analyses have shown that multiple genetic alterations are involved in the genesis and progression of urothelial cell carcinoma of the bladder[27]. Among these alterations, deletions of chromosome 9p (9p21 - p14, p15 and p16 proteins) or 9q (9q13-31, 9q32-33 and 9q34) are the most frequent, suggesting that the loss of tumour suppressor genes lead to cell uncontrolled proliferation and increasing in the DNA amount[28]. On the other hand, these 9q altered regions are close to the 9q34.1-34.2, the mapped area for the ABH genes[11].

Compared with flow cytometry, image cytometry has limitations in distinguishing cell populations with small alterations in DNA content, like near-diploid populations, however, it requires a lower number of cells allowing the study of small tumour sample[29]. Furthermore, microscopic selection of tumour cells allows the rejection of artefacts as well as the detection of aneuploid cells that may represent a small proportion of the whole tumour cell population. In the present study the DNA content was assessed by ima-

Table 4 – Discriminatory distribution of Ki-67 and A.B.H. blood group antigen expression for 5cER

			5cER			
Ki67			Without	With	**Total**	**p**
>18%	**A.B.H**	Expression	2	1	3	
		Partial Loss	2	6	8	
		Total Loss	4	8	12	0.282
		NS		4	4	
	Total		8	19	27	
1-18%	**A.B.H.**	Expression	2	3	5	
		Partial Loss	4	2	6	
		Total Loss	5	6	11	0.590
		NS	1		1	
	Total		12	11	23	
negative	**A.B.H.**	Expression		6	6	
		Partial Loss	1	6	7	
		Total loss	4	25	29	0.008
		NS	2		2	
	Total		7	37	44	

ge cytometry. Regarding all studied DNA parameters (DNA ploidy, DNA histogram type and percentage of 5cER cells) similar trends were observed when compared with grade and stage. All TaG1 urothelial tumours were DNA diploid, being the incidence of the aneuploid pattern (ploidy and DHTs) and the 5cER value increased with grade and/or stage. Among these DNA content related variables, 5cER was shown to be the variable that represents the less time consuming procedure if we previously set-up the morphometric image filter to select only these aneuploid cell nuclei. This parameter has been showed to be a useful tumour marker in bladder cancer [49].

Previous studies have demonstrated an association between cell proliferation and tumour grade, stage, recurrence and progression in bladder carcinoma[30-33]. In our series, the majority of G3 tumours (T1 – 6 cases and T2/T4 – 28 cases) showed no proliferative cells, however, these cases were aneuploid and lost the ABH antigens. Similar results where observed by Baithun et al [34], who found a distinct genetic and kinetic profile (slower cell turnover) in musclenvasive bladder cancer. Blanes et al[35], studying kinetic parameters like mitotic figures counting, Ki-67 LI and proliferative rate assessed by image cytometry in same bladder carcinomas, observed a lower proliferation rate in deep compartment of tumours. This lower rate could be a consequence of the absence of tumour suppressor gene alterations, as referred by Diaz--Cano et al [36].

Loss of blood group antigen was an early event in our series of secretor superficial low-grade tumours of urinary bladder. Juhl et al[37] also observed a small number of superficial bladder tumours exhibiting a normal blood group antigen expression. In their work, Ornotof et al[38] concluded that in normal and malignant urothelium ABH glycosylation is regulated at the mRNA level. This mechanism is associated with cell proliferation that may trigger down the regulation of ABH mRNA[38]. In our series we did not show a significant association between high Ki-67 labelling index and loss of ABH expression. Therefore, alteration of glycosyltranferase encoding genes may occur, as suggested by Orlow et al[39].

In this series we verified that early tumours (Ta/G1) had high proliferative index and blood group antigens loss of expression. Nevertheless, these tumours remained diploid. Therefore, we can say that, indeed, chromosome 9 loss (partial or entire) is an early event and is accompanied by cell proliferation. When we analysed more invasive tumours, the proliferation rate is diminished, the ABH antigens expression loss remains, but they are no longer diploid. According to

our results, aneuploidy i.e., DNA content alteration is not an early event. During the progression of urothelial cell carcinoma of the bladder there seems to be a selection of those cells with gains of genetic material, conferring the DNA aneuploid status. The alteration in the parameters studied were usually related with worse prognosis and consequently determining more aggressive therapy[5, 7, 14, 20]. The loss of ABH antigen immunoreactivity is a surrogate marker of urothelial cancer cells aberrant glycosylation. This suggests that adjuvant immunization-based strategies can be used to mobilise the immune system against criptic carbohydrate antigens, displayed on the surface of those cancer cells.

Acknowledgements

The authors are indebted to CFICS, Ministério de Saúde de Portugal, project nº 222/01 for the financial support.

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Artigo #3: CIS is a surrogate marker of genetic instability and field carcinogenesis in the urothelial mucosa.

Palmeira C, Lameiras C, Amaro T, Lima L, Kock A, Lopes C, Oliveira PA, Santos L

Urol Oncol 2010 (em publicação).

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Urologic Oncology: Seminars and Original Investigations xx (2009) xxx

UROLOGIC ONCOLOGY

Original article

CIS is a surrogate marker of genetic instability and field carcinogenesis in the urothelial mucosa

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Received 5 May 2009; received in revised form 13 July 2009; accepted 13 July 2009

Abstract

Objective: To investigate whether carcinoma in situ (CIS) lesion could be considered a surrogate marker of urothelium genetic instability and field carcinogenesis or not, we evaluated DNA content, p53 overexpression, and proliferative index (Ki-67 expression) in primary tumor, in tumor-adjacent mucosa, and distant urothelial mucosa with and without presence of CIS.

Patients and methods: A retrospective study in radical cystectomy specimens from 49 patients was carried out. All the lesions present in each cystectomy specimen were studied, including the tumor area and the adjacent mucosa (AM). Whenever possible, the distant mucosa (DM) was also studied. When CIS was detected, this lesion and the surrounding normal mucosa were also studied. The 49 tumor areas included high grade papillary urothelial carcinoma (HGP) in 19 cases (38.8%) and invasive urothelial cell carcinomas in 30 cases (61.2%). The nuclear DNA content of cancer cells was evaluated using image cytometry allowing the determination of the DNA ploidy and 5cER parameters. The p53 and Ki-67 immunoexpression was evaluated by immunohistochemistry.

Results: CIS lesions were observed in the AM and DM of both tumor groups: 15.8% and 15.4% in AM and DM, for each one of them, in HGP group and 26.7% and 22.2% in AM and DM, for each one of them, in invasive tumors group. In CIS lesion aneuploid DNA content, p53 overexpression and high proliferative labeling index were observed. The so-called normal mucosa (AM and DM) with and without focus of CIS lesions were compared for genetic instability and molecular alterations profile. Statistical differences were observed between the normal mucosa with and without CIS: the so-called normal mucosa areas with focus of CIS revealed significantly higher frequencies of DNA content alterations, p53 overexpression, and higher proliferative index. These differences were significantly different in the invasive UCC group, but this profile it is also present in HPG group.

Conclusion: This study points out that CIS is a marker of genetic instability of the urothelium mucosa. The CIS surrounding morphologically normal urothelium showed a high frequency of abnormal DNA content, with high percentage of clear aneuploid cells (high 5cER), p53 mutated protein expression, and a proliferative status underlying a field carcinogenesis. These alterations in normal mucosa were not found when CIS was not present. © 2009 Elsevier Inc. All rights reserved.

Keywords: Bladder cancer; CIS; DNA content; p53 immunoexpression; Ki-67 immunoexpression; Genetic instability

1. Introduction

Carcinoma in situ (CIS) is a high-grade lesion of the bladder that is confined to the urothelium mucosa, and is included in the superficial urothelial cell carcinoma group (sUCC). It is characterized by flat, disorderly proliferation of cells with marked cytologic abnormalities, and comprises about 10% of all bladder cancers [1].

The clinical experience showed that patients presenting with isolated or concomitant CIS lesions (T1) have a high risk of disease progression to a muscle invasive stage [2]. If

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1078-1439/09/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.urolonc.2009.07.022

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transurethral resection (TUR) is the single treatment used, nearly one-half of the patients with CIS progress to invasive disease within 5 years of diagnosis [3].

According to the European Association of Urology (EAU) guidelines for bladder cancer [4], primary CIS confined to the bladder is treated with TUR and intravesical bacillus Calmette-Guerin (BCG) immunotherapy. Despite the complete response rate achieved (83%–93%) [5,6], as many as 20% of patients will ultimately die of metastatic disease, and a significant proportion will develop both intravesical and extravesical recurrences [1]. Therefore, the high risk of progression to muscle invasive tumors at the time of clinical diagnosis (55%) and the high incidence of bladder cancer recurrence and progression after BCG treatment in this group of patients promote an intense discussion about: (1) Is it reasonable to propose early cystectomy as primary treatment in patients with high grade papillary urothelial carcinoma (HGP) with associated CIS? (2) Should one advocate immediate cystectomy in patients with HGP with associated CIS after BCG failure? (3) Is CIS lesion a surrogate marker of urothelium genetic instability and field carcinogenesis?

In order to answer the last question, we evaluated DNA content, p53 immunoreactivity, and proliferative index (Ki-67 expression) with the objective to identify genetic instability and cell homeostasis deregulation in primary, in tumor-adjacent mucosa, and in distant urothelial mucosa with and without presence of CIS.

2. Patients and methods

From a series of 735 patients with urothelial cell carcinoma admitted and treated at the Instituto Português de Oncologia do Porto, Portugal between 1989 and 1996, we studied 49 consecutive radical cystectomy (RC) specimens with suitable archival material to image cytometry study with patient' consent. None of these patients had received prior neoadjuvant chemotherapy. Indications for RC, in this series, were invasive tumors, recurrent and/or multifocal high risk non-muscle-invasive bladder cancer, and, in few cases, life saving cystectomy (severe hemorrhage/perforation). Clinical data were collected from patients' records.

All the lesions present in each cystectomy specimen were studied, including the tumor area and the adjacent mucosa (AM). Whenever possible, the distant mucosa (DM) was also studied and was ureter mucosa. From each multifocal case, a tumor fragment representative of the major clinical diagnostic, an adjacent, and DM fragments were selected and placed together in the same paraffin block.

All the histologic samples were reviewed by a pathologist with expertise in genitourinary pathology. Pathologic staging was performed according to the American Joint Committee on Cancer system [7] and histologic classification and grading using the criteria from the 2004 World Health Organization (WHO) guidelines [8,9]. Pathologic alterations in adjacent and distant mucosa were also classified according with 2004 WHO classification [8]. Mucosa without histopathologic lesions was defined as "so-called normal mucosa."

The 49 tumor areas studied include 19 (38.8%) HGP and 30 (61.2%) invasive UCCs. The AM, in all cases, was also studied. Distant mucosa was evaluated in 31 cases: 13 in HGP group and 18 in invasive carcinoma group.

In order to evaluate the genetic instability and molecular alterations profile attributable to CIS lesion presence, we considered two groups of patients, one with CIS and the other without CIS lesion. In both groups, the so-called normal mucosa in the adjacent and distant mucosa was studied, regarding the biological variables included in the present work.

2.1. DNA content analysis

The nuclear DNA content of cancer cells was evaluated using the CAS 200 Image Analysis System (Cell Analysis Systems, Inc., Elmhurst, III). The image cytometry method and the DNA histogram analysis used were previously described in detail [10] and will be summarized here. The image system was first calibrated using the control slide, which contained rat hepatocytes with a known quantity of DNA. Twenty to 30 lymphocytes and a minimum of 100 intact non-overlapping urothelial nuclei were measured and analyzed for each case, using the Quantitative DNA Analysis software program (Cell Analysis System, Inc.). The G0/G1 peak was visually identified in each DNA histogram, and the mean standard deviation (SD) and the coefficient of variation (CV) values were calculated. The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of mean DNA content of the urothelial G0/G1 peak divided by the mean DNA content of the resting diploid lymphocytes G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with values above 5n, clear aneuploid cells. Lesions were considered aneuploid only if a separate G0/G1 peak was distinguishable on the histogram, and it differed from the reference lymphocyte population by > 2 SD. A DNA diploid lesion showed a single distinct G0/G1 peak with a DI within 2SD of the control lymphocytes and usually with $<1\%$ of 5cER.

2.2. Immunohistochemistry

The immunoexpression of p53 and Ki-67 was evaluated according to a previously described method [11] and using a standard avidin-biotin peroxidase method. The primary antibodies D07 (1:50 dilution, DAKO, Carpinteria, CA) and PAb-240 (1:50 dilution, NeoMarkers, Lab Vision, Fremont, CA) for p53 expression, and MIB1 (1:50 dilution, Novocastra, Newcastle, UK) for Ki-67 were used.

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Table 1
Clinical and histopathologic characteristics for all the 49 patients

Characteristics	No. patients (%)
Sex	
Female	13 (26.5)
Male	36 (73.5)
Pathological stage	
pT1	17 (34.6)
pT2	16 (32.7)
pT3-T4	16 (32.7)
Histology classification	
HGP	19 (38.8)
INV	30 (61.2)
Concomitant CIS lesions	
Presence	12 (24.5)
Absence	37 (75.5)
Multiple tumors	
Presence	11 (22.5)
Absence	38 (77.5)

HGP = high grade papillary urothelial carcinoma; INV = invasive urothelial carcinoma.

Paraffin-embedded tissues known to express nuclear p53 (colon carcinoma) and known to have high proliferation rate (lymphoma) were used for titration and positive controls. Negative controls were performed replacing the primary antibody for 2.5% bovine serum albumin in phosphate-buffered saline.

DO7 mouse monoclonal antibody reacts with an epitope in the N-terminal extreme of the p53 protein, located between the amino acids 19 and 26, and recognizes both mutant and wild type proteins. PAb-240 identifies mutant p53 protein for those mutations occurring between codon 212 and 217.

2.3. Immunohistochemical scoring

Immunohistochemical analysis was done by 2 independent observers (TA and LS) in a blind fashion, without knowledge of the clinical data. All conflicts in scoring between these two observers were re-evaluated together with a third review (CL). The evaluation method was also described previously [11]. The entire tissue section was screened to find the region with maximum fraction of positive and contiguous stained nuclei for p53 and the region with maximum fraction of positivity for Ki-67. The percentage of positive stained nuclei was scored in this region using the 40× microscope objective. At least 200 malignant cells were scored in each case. The cut-off values used for p53 and Ki-67 were >18% and >32%, respectively, based on studies reported previously [11].

2.4. Statistical analysis

All statistical analysis was performed with SPSS 15.0 software (SPSS Inc., Chicago, IL). First a descriptive study was done for all variables included in the study. Pearson's χ^2 and Fisher's exact (when $n < 5$) tests were used to compare categorical variables. $P < 0.05$ was accepted as statistically significant.

3. Results

Clinical and histopathologic characteristics for the patients included in this study are listed in Table 1. The median age was 73 years (range, 28–83 years). For each of the 49 tumors included in the present study, the tumor area, the respective AM, and DM were microscopically screened, and histologic characterization was done. The histopathologic alterations found in the AM and DM of the HGP and invasive UCC are presented in Fig. 1. CIS lesions were observed in the AM and DM of both tumor groups: 15.8% and 15.4% in AM and DM, respectively, in HGP group and 26.7% and 22.2% in AM and DM, respectively, in invasive tumors group.

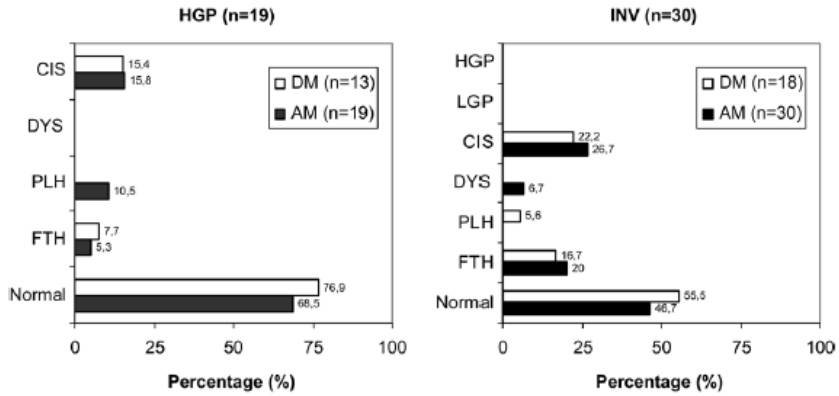

Fig. 1. Histologic characterization of the AM and DM in the HGP and invasive (INV) urothelial cell carcinomas. Normal: normal urothelium; FTH: flat urothelial hyperplasia; PLH: papillary urothelial hyperplasia; DYS: dysplasia; CIS: carcinoma in situ.

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Table 2
Alterations in CIS lesions present in the adjacent mucosa (AM) of high grade papillary UCC (HGP) and invasive UCC (INV)

Variables	HGP $n = 3$ No. cases (%)	INV $n = 8$ No. cases (%)	P
DNA ploidy			0.521
Diploid	0 (0)	1 (12.5)	
Aneuploid	3 (100)	7 (87.5)	
5cER			0.521
Without	0 (0)	1 (12.5)	
With	3 (100)	7 (87.5)	
p53 (DO7)			0.898
Negative	2 (66.7)	5 (62.5)	
Positive	1 (33.3)	3 (37.5)	
p53 (Ab-240)			0.898
Negative	2 (66.7)	5 (62.5)	
Positive	1 (33.3)	3 (37.5)	
Ki-67			0.125
Negative	0 (0)	4 (50.0)	
Positive	3 (100)	4 (50.0)	

Comparing the CIS lesions observed in the AM near by HGP and invasive tumors, for biological variables studied no significant differences were observed (Table 2). In distant mucosa (DM), similar results were found in CIS lesions near by HPG or invasive urothelial bladder carcinoma (Table 3).

When we compared CIS lesions (itself) detected in AM and DM, again, no statistical differences were observed for biological variables studied (Table 4). CIS lesions revealed aneuploid DNA content, p53 overexpression, and high proliferative labeling index, independently of the localization and associated tumor. The same biological pattern observed in the CIS lesion was also observed in the so-called normal mucosa adjacent to CIS lesions (Fig. 2).

Table 3
Alterations in CIS lesions present in the distant mucosa (DM) of high grade papillary UCC (HGP) and invasive UCC (INV)

Variables	HGP $n = 2$ No. cases (%)	INV $n = 4$ No. cases (%)	P
DNA ploidy			0.333
Diploid	1 (50.0)	0 (0)	
Aneuploid	1 (50.0)	4 (100)	
5cER			0.333
Without	1 (50.0)	0 (0)	
With	1 (50.0)	4 (100)	
p53 (DO7)			0.540
Negative	1 (50.0)	1 (25.0)	
Positive	1 (50.0)	3 (75.0)	
p53 (Ab-240)			0.540
Negative	1 (50.0)	1 (25.0)	
Positive	1 (50.0)	3 (75.0)	
Ki-67			0.540
Negative	1 (50.0)	1 (25.0)	
Positive	1 (50.0)	3 (75.0)	

Table 4
Alterations in CIS lesions present in the adjacent mucosa (AM) and distant mucosa (DM)

Variables	AM $n = 11$ No. cases (%)	DM $n = 6$ No. cases (%)	P
DNA ploidy			0.650
Diploid	1 (9.1)	1 (16.7)	
Aneuploid	10 (90.9)	5 (83.3)	
5cER			0.650
Without	1 (9.1)	1 (16.7)	
With	10 (90.9)	5 (83.3)	
p53 (DO7)			0.229
Negative	7 (63.6)	2 (33.3)	
Positive	4 (36.4)	4 (66.7)	
p53 (Ab-240)			0.229
Negative	7 (63.6)	2 (33.3)	
Positive	4 (36.4)	4 (66.7)	
Ki-67			0.900
Negative	4 (36.4)	2 (33.3)	
Positive	7 (63.6)	4 (66.7)	

Therefore, the so-called normal mucosa (AM and DM) with and without focus of CIS lesions, were compared for genetic instability and molecular alterations profile. Tables 5 and 6 describe the results observed in the adjacent and distant normal mucosa, in the invasive and HGP urothelial bladder cancer cases, respectively. Statistical differences were observed between the normal mucosa with and without CIS: the so-called normal mucosa areas adjacent to CIS revealed significantly higher frequencies of DNA content alterations, p53 overexpression and mutations, and higher proliferative index. These differences were especially evident in the invasive UCC group. In HGP tumors, the adjacent so-called normal mucosa of all cases with CIS revealed aneuploidy and high proliferative index, however, the frequency of p53 overexpression occurred only in 33.3% cases. In distant mucosa (two cases), 50% showed aneuploidy, high proliferative index, and p53 overexpression.

These findings enhanced the observation referred to above that the molecular alterations found in CIS lesion were also observed in the surrounding so-called normal mucosa.

4. Discussion

Our study points out that CIS is a marker of genetic instability of the urothelium mucosa. The CIS surrounding normal mucosa showed a high frequency of abnormal DNA content, with high percentage of clear aneuploid cells (high 5cER), p53 mutated protein expression, and a proliferative status underlying a field carcinogenesis. These alterations in normal mucosa were not found when CIS was not present.

The study of urothelial genetic alterations related to bladder cancer due to DNA content changes, p53 immunoreactivity, and high proliferative labeling index have been

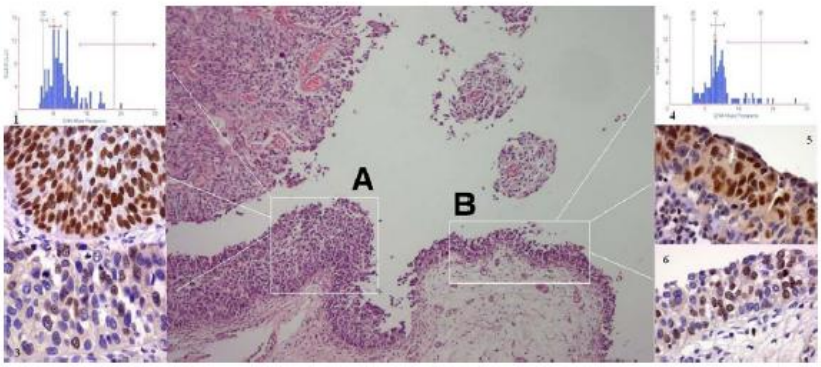

Fig. 2. Biological pattern observed in the CIS and in the so-called normal urothelium without CIS, regarding DNA content alterations, p53 overexpression, and proliferative labeling index. Stained tissue section showing the HGP and AM with and without CIS (H and E, 40×). (A) 1, 2, and 3: CIS; (B) 4, 5, and 6: so-called normal urothelium without CIS; 1 and 4: aneuploid DNA content with 5cER; 2 and 5: p53 DO7 and Ab-240, respectively, overexpression (400×); 3 and 6: Ki-67 immunoexpression (400×).

the main molecular studies in human urothelial carcinogenesis [12] and animal experimental induced urothelial carcinogenesis in our research [10,13–16]. In both models, we found a concomitant increase in the frequency of the aneuploid pattern, abnormal p53 expression, and high Ki-67 index in the UCC with higher malignant potential and aggressiveness, namely CIS, high grade papillary and muscle-invasive tumors. Therefore, these alterations found in the three biological variables represent a high level of genetic instability related with urothelium carcinogenesis process. This similar molecular profile found between CIS and invasive urothelial carcinomas was also observed by others [17,18]. In rodent CIS urothelial lesions induced by N-butyl-N-(4-hydroxybutyl) nitrosamine [14] or induced by repeated intravesical instillation with mitomycin C [16], we found identical molecular alterations. The results of the present work are in agreement with the CIS gene expression signature concept described by Dyrskjøt and colleagues [2,19]. These authors described a CIS classifier with a group of 16 genes that are mainly related with the regulation pathways of growth factors receptors, cell cycle control, and apoptosis regulation. According to these authors, a CIS genetic profile is present in the normal-appearing urothelium and in neighboring UCC [2]. We may assume that CIS is a surrogate marker of urothelium genetic instability and field carcinogenesis. Höglund and colleagues [20], proposed the field-first-tumor-later model, in which nonmalignant but genetically modified cells spread through the epithelium and

Table 5
Comparison of genetic instability and molecular alterations profile in the so-called normal mucosa (AM and DM) according to the presence of CIS lesions in the invasive UCC group (INV)

Variables	Tumour area	AM (normal mucosa)		*P*	DM (normal mucosa)		*P*
	INV *n* = 30 No. cases (%)	Without CIS *n* = 14 No. cases (%)	With CIS *n* = 8 No. cases (%)		Without CIS *n* = 10 No. cases (%)	With CIS *n* = 4 No. cases (%)	
DNA ploidy				0.006			0.005
Diploid	2 (6.7)	11 (78.6)	1 (12.5)		9 (90)	0 (0)	
Aneuploid	28 (93.3)	3 (21.4)	7 (87.5)		1 (10)	4 (100)	
5cER				<0.001			0.001
Without	2 (6.7)	13 (92.9)	1 (12.5)		10 (100)	0 (0)	
With	28 (93.3)	1 (7.1)	7 (87.5)		0 (0)	4 (100)	
p53 (DO7)				0.117			0.041
Negative	15 (50.0)	13 (92.9)	5 (62.5)		9 (90)	1 (25)	
Positive	15 (50.0)	1 (7.1)	3 (37.5)		1 (10)	3 (75)	
p53 (Ab-240)				0.036			0.011
Negative	17 (56.7)	14 (100)	5 (62.5)		10 (100)	1 (25)	
Positive	13 (43.3)	0 (0)	3 (37.5)		0 (0)	3 (75)	
Ki-67				0.039			0.041
Negative	8 (26.7)	13 (92.9)	4 (50)		9 (90)	1 (25)	
Positive	22 (73.3)	1 (7.1)	4 (50)		1 (10)	3 (75)	

AM = adjacent mucosa; DM = distant mucosa.

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Table 6
Comparison of genetic instability and molecular alterations profile in the so-called normal mucosa (AM and DM) according with the presence of CIS lesions, in the high grade papillary UCC group (HGP)

Variables	Tumour area	AM (normal mucosa)		P	DM (normal mucosa)		P
	HGP $n = 19$ No. cases (%)	Without CIS $n = 13$ No. cases (%)	With CIS $n = 3$ No. cases (%)		Without CIS $n = 10$ No. cases (%)	With CIS $n = 2$ No. cases (%)	
DNA ploidy				0.063			0.345
Diploid	7 (36.8)	9 (69.2)	0 (0)		8 (88.9)	1 (50)	
Aneuploid	12 (63.2)	4 (30.8)	3 (100)		1 (11.1)	1 (50)	
5cER				0.002			0.182
Without	9 (47.4)	13 (100)	0 (0)		9 (100)	1 (50)	
With	10 (52.6)	0 (0)	3 (100)		0 (0)	1 (50)	
p53 (DO7)				0.188			0.455
Negative	14 (73.7)	13 (100)	2 (66.7)		8 (80)	1 (50)	
Positive	5 (26.3)	0 (0)	1 (33.3)		2 (20)	1 (50)	
p53 (Ab-240)				0.188			0.167
Negative	17 (89.5)	13 (100)	2 (66.7)		10 (100)	1 (50)	
Positive	2 (10.5)	0 (0)	1 (33.3)		0 (0)	1 (50)	
Ki-67				0.002			0.167
Negative	7 (36.8)	13 (100)	0 (0)		10 (100)	1 (50)	
Positive	12 (63.2)	0 (0)	3 (100)		0 (0)	1 (50)	

AM = adjacent mucosa; DM = distant mucosa.

eventually develop into overt tumors independently, as our results may suggest.

According to the European Organization for Research and Treatment of Cancer (EORTC) bladder cancer risk groups, patients with a non-muscle-invasive tumor with a concomitant CIS lesion have a high risk of progression and recurrence [4]. BCG is the standard treatment for these patients, yielding an initial complete response rate of 83% to 93% [5,6]. However, approximately 50% of patients develop recurrent disease with muscle invasion or extravesical tumor [5,21]. Gofrit and colleagues [22] indicated that patients with pure or concomitant CIS had a similar prognosis, which suggests, therefore, that CIS itself is the major determinant of patients' prognosis and not other past or concomitant bladder tumors.

In spite of the consensus regarding the benefit of a RC in these patients, this is still a controversial subject [3,4,23–28]. Even the optimal timing to RC has not yet been established for clinical use [23,29–32]. The importance of the presence of CIS as a factor that could help to distinguish between cases requiring early RC and those suitable for organ preservation has been evaluated by some studies and also showed contradictory results [30].

We understand that superficial bladder cancer with CIS should not be a local disease but a diffuse abnormality of the urothelium. Therefore, the question is: In the presence of a HGP with concomitant CIS, should we treat the tumor or field carcinogenesis?

Considering that this high risk superficial bladder cancer is indeed a "field disease," we think that adequate therapy strategy should include a "field treatment" approach, in other words, performing a radical removal of the genetically unstable urothelium or the maintenance of immunotherapy by intravesical BCG instillations.

The aim of the present study was not to answer this question. Our purpose was to contribute to a better understanding of the molecular profile of CIS and surrounding urothelial mucosa. Therapy that can adjust better to the biology of this lesion will be the more efficient.

5. Conclusion

Our results suggest clearly that CIS is a surrogate marker of a high potential of malignant transformation in the surrounding normal urothelium and indicates that the field cancerization process is going on.

Acknowledgments

The authors are grateful to Eng. D. Sanders, Portuguese Institute of Oncology, for the English version.

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Modelo Animal

Artigo #4: Evaluation of DNA content in preneoplastic changes of mouse urinary bladder induced by N-butyl-N-(4-hydroxybutyl) nitrosamine.

Oliveira PA, Palmeira C, Lourenço LM, Lopes CA.

J Exp Clin Cancer Res. 2005; 24: 609-616.

J. Exp. Clin. Cancer Res., 24, 4, 2005

Evaluation of DNA Content in Preneoplastic Changes of Mouse Urinary Bladder Induced by N-Butyl-N-(4-Hydroxybutyl) Nitrosamine

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Using image cytometry analysis we analysed the deoxyribonucleic acid content of preneoplastic lesions induced in C3H/He female mice. Female mice (n=30) were given 0.05% BBN in their drinking water for 4, 8 and 12 weeks, and were then euthanized. At the 4th week 90% hyperplasias, 90% dysplasias and 10% papillary tumours developed selectively in the bladder, after 8 weeks mice developed 80% hyperplasias and 80% dysplasias and 12 weeks we identified 70% hyperplasias, 70% dysplasias, 10% papillary tumours and 20% squamous metaplasia. Among the ones who developed simple hyperplasia, 58.3% were diploid and 41.7% were aneuploid. In the dysplasia lesions 29.2% were diploid and 70.8% were aneuploid (p=0.041). All papillary tumours were aneuploid and all epidermoid metaplasias were diploid. These results suggest that aneuploidization, DNA content alteration of preneoplastic lesions, might be considered as a prognostic factor.

Key Words: Bladder carcinogenesis, DNA ploidy, Mice, Image cytometry analysis, BBN

The development of urinary bladder cancer is a multistep process; preneoplastic lesions occur before *in situ* and invasive carcinoma, and persist for many years in several parts of the urothelium, providing the opportunity for a repeated formation of neoplasms. Also, specific studies of DNA content in preneoplastic lesions have not been performed.

Many experimental models using rodents have been established to study the different stages of urinary bladder chemical carcinogenesis (1-5). The N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) induced model is one of them, and widely used. BBN is a genotoxic compound with a potent and selective organotropic carcinogenic activity, inducing the development of preneoplastic and neoplastic lesions in the urothelium of mice, rats, hamsters and dogs (6-11). BBN needs metabolic activation in the liver and urothelium as well as repeated doses so as to exert a carcinogenic effect (12,13). The histopathological spectrum of lesions induced by BBN in experimental models are identical to those observed in the human bladder, thus providing a suitable animal model for human disease (2). The comprehension of urothelium carcinogenesis in the mouse can contribute to a better understanding of the bladder neoplastic transformation.

It has long been known that carcinogenesis is frequently associated with chromosomal alterations, genomic changes, measurable differences in deoxyribonucleic acid (DNA) content analysis and variations in the cell cycle of neoplastic cells (14). The last two variables can be measured quantitatively and reliably by image cytometry analysis (ICM) and flow cytometry. ICM consist in the measurement of DNA cell content using a non fluorescent compound which attaches to DNA: the integrated optical density measured in each stained nuclei is proportional to the amount of DNA present in the nuclei. This kind of measurement provides two different types of biological information. Firstly, information on the existence or absence of clonal abnormalities in the DNA cell content, i.e, DNA aneuploidy. Secondly, it allows us to analyse the distribution of a certain cell population through the different phases of the cell cycle (15). DNA cell quantification using ICM techniques have provided valuable information about biology of pathological cells (16). Regarding bladder tumors, several studies have shown that DNA ploidy analysis can help predict neoplastic progression (17-21).

Factors affecting the carcinogenesis process, such as initiation and promotion in the BBN model have been examined, but the biological characteristics associated with this process are relatively unexplored. To our knowledge, combination studies using ICM analysis have not been reported in a mouse bladder tumor model. The present experiment was performed to study the sequence of urothelium morphological changes associated with oral administration of BBN to female mice. A combined histopathological and ICM analysis was used to study this evolution process of bladder carcinogenesis.

Materials and Methods

Animals and chemicals. Forty-five, six weeks old female mice of the strain C3H/He (24 to 30gm) were obtained from the Instituto Gulbenkian (Oeiras, Portugal). They were randomly housed in three groups of ten and three groups of five animals (controls) per plastic cage with hard wood chips for bedding in a room with a targeted temperature of 23+/-2°C, 55+/-10% humidity and a 12 hours light/day. Food, standard mouse chow, and tap water were available ad libitum at all times during the study. They were submitted to an acclimatization period of 2 weeks. They did not receive any treatment prior to the study.

Experimental design and collection of the bladder. BBN was obtained from Tokyo Kasey Kogyo (Tokyo, Japan) and administered in drinking water in opaque bottles at a concentration of 0.05% to the groups of ten animals, for 4, 8 and 12 weeks. The control groups were given tap water throughout the experiment. Bedding and cages were changed twice per week. The experimental designed is presented in Fig. 1. During the experiment a daily health check was performed and body weights were measured twice per week. Consumption of food and water were measured at the same time during which body weights were determined.

Mice were sacrificed at 4, 8 and 12 weeks of BBN exposition, the bladders were immediately removed and complete autopsies were carefully conducted. The urinary bladders were inflated *in situ* by injection of 0.1ml of a fixative solution of 10% phosphate-buffered formalin, ligated around the neck to maintain proper distension and then were immersed in the same solution for 24 hrs. After fixation, each urinary bladder was carefully examined to detect the presence of lesions, and transected to yield an anterior and posterior cup shaped area. After longitudinal bisection and embedding in paraffin wax, 2µm sections were cut and stained with hematoxylin and eosin for light microscopic examination.

Quantification of DNA content. Urinary bladder sections from paraffin-embedded blocks were cut at 6µm and deparaffinized and rehydrated. The slides were then Feulgen-stained with the CAS DNA staining kit (Cell Analysis Systems, Elmhurst, III), according to the manufacture´s instructions. Briefly, the sections were hydrolyzied in 5N HCl solution for 60 minutes and then transferred to the CAS DNA stain solution for 1 hour. After staining, the slides were placed in three consecutive CAS rinse solutions for 30 seconds, 5 minutes, and 10 minutes, respectively. The slides were then washed in deionized water and placed in acid alcohol solution for 5 minutes. Finally, the slides were dehydrated and mounted. The Feulgen reaction produced a blue staining of nuclear DNA reflecting stoichiometric binding of the stain to the DNA (22). CAS staining of rat hepatocyte was used as a control slide. These cells, having a known quantity of DNA, were used as external control, allowing the instrument calibration prior to DNA image analysis. The nuclear DNA content of cells was measured with the CAS 200 image analysis system (Cell Analysis Systems, INC, Elmurst, III) using the quantitative DNA analysis software program (Cell Analysis System). This analysis system is well suited for DNA content evaluation in human cells. However, if the diploid value of the specimens studied is different from human diploid cells, an "internal control" must be used, such as lymphocytes (22,23). For this reason in each lesion, in addition to a minimum of 100 non-overlaping and well preserved urothelial nuclei, 20 to 30 normal lymphocytes were measured. The resultant DNA histograms were analysed by previously described methods (19,24). **For each lesion, G0/G1 peak visually identified, mean, standard deviation (SD) and coefficient of variation (CV) values were calculated.** The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of the mean DNA content of the urothelial G0/G1 peak divided by the mean DNA content of the resting diploid lymphocytes G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with DNA values above 5n. Lesions were considered aneuploid only if a separate G0/G1 peak was distinguishable on the histogram and its DI differed from the reference lymphocyte population by more than 2SD, mean standard deviation. A DNA diploid lesion

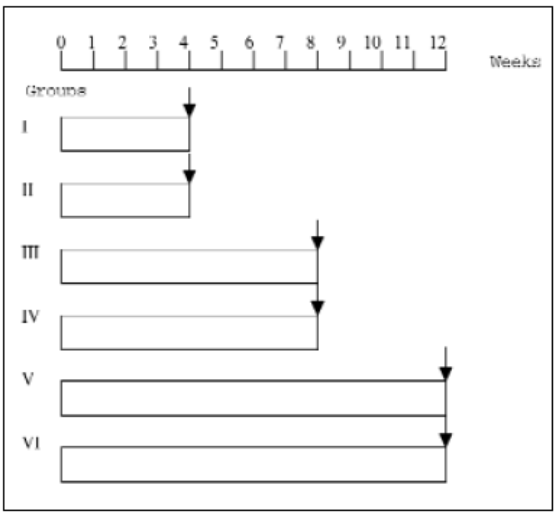

Fig. 1 - Experimental design. Grey: 0.05% BBN administered in the drinking water; white: only tap water as drinking water; arrows: animal sacrifice time points.

showed a single distinct G0/G1 peak with a DI within 2SD from the control lymphocytes and usually with less than 1% of 5cER.

Histological classification. The sections were examined for the presence of epithelial and mesenchymal lesions. The histopathological types of lesions induced by BBN were classified according to the classification system previously proposed for the mouse, based upon the WHO criteria for human bladder (25). Briefly, the lesions were classified as follows: simple hyperplasia, dysplasia, papillary tumor; squamous cell metaplasia.

Statistical analysis. The statistical analysis was done using the SPSS 8.0 software. For all variables included in this study a descriptive analysis was performed. The differences for body weights and food ingestion between the groups at each experimental moment were analysed by ANOVA. The correlation between the type of lesion observed and number of weeks of treatment and DNA results were analysed by chi-square and Fisher's exact tests. The difference was considered statistically significant at $p<0.05$.

Results

Clinical observation did not reveal any abnormalities among the groups and no deaths occurred during the treatment period. No significant differences in the growth curves of the mice were observed in any groups. Regarding BBN effects, no significant growth retardation was observed during the administration period. Food and water consumption between groups was similar, not significantly different.

No relevant macroscopic changes were seen in the liver, lung, kidney and gastrointestinal tract. However, in the urinary bladder epithelium, histological changes were observed and classified into simple hyperplasia, dysplasia, papillary tumour and squamous cell metaplasia; the data are summarized in Table I.

The urothelium of the urinary bladder in control groups II, IV and VI revealed no histological changes.

Normal bladder epithelium showed three distinct cell layers. A most superficial layer was composed of relatively large cells, the intermediate cell layer consisted predominantly of a single cell layer, the basal layer with smaller cells, in all layers cells were polymorphic and underwent shape transformation (Fig.2).

Simple hyperplasia consisted of focal or multifocal thickening of transitional cell lining, with or without cellular atypia (Fig.3). In dysplastic lesions we identified cellular atypia, nuclear pleomorphism, cytoplasmic basophilia, and mitotic activity (Fig.4). In papillary tumours we observed papillary growth with proliferating connective tissue. The cells were well organised and similar in appearance but more uniform in size with slightly more basophilic cytoplasm. Mitotic activity was low (Fig.5). In squamous cell metaplasia we observed a replacement of transitional epithelium by squamous cells, with a focal or multifocal distribution. Surface cells were keratinized and contained keratohyaline granules, nuclei were round, oval or flattened. We also observed desquamation of the cornified material (Fig.6).

ICM was successfully performed on 24 hyperplasias, 24 dysplasias, 2 papillary tumours and 2 squamous metaplasias. Two representative histograms obtained by ICM are shown in Figures 7 and 8. Table II summarizes the DNA ploidy of the different lesions observed after 4, 8 and 12 weeks of BBN exposition. Despite the time of BBN exposition the frequency of a DNA aneuploid pattern increased with the degree of tissue transformation, with the lowest percentage in the hyperplasia lesions and the highest in the papillary tumors. This difference was statistical significant between the two larger groups of lesions, hyperplasias and dysplasias ($p=0.041$) (Table III). All papillary tumours were aneuploid and all squamous cell metaplasia were diploid.

Table I - Incidence of urinary bladder lesions in C_3H/He mice with and without BBN 0.05% BBN treatment

			Number of animals with lesions (%)			
Group	Treatment Weeks	Number of mice	Hyperplasia	Dysplasia	Papillary tumours	Squamous cell metaplasia
I	BBN 4W	10	9(90)	9(90)	1(10)	0
III	BBN 8W	10	8(80)	8(80)	0	0
V	BBN 12W	10	7(70)	7(70)	1(10)	2(20)
II	CTRL 4W	5	0	0	0	0
IV	CTRL 8W	5	0	0	0	0
VI	CTRL 12W	5	0	0	0	0

W-Weeks; CTRL-Control groups.

Fig. 2 - Normal urothelium of mouse (H&E, 100x).

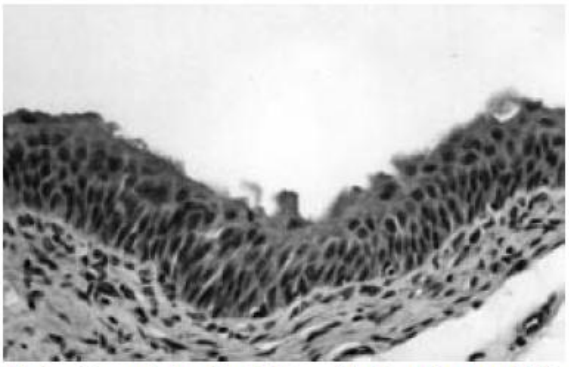

Fig. 3 - Hyperplasia of the bladder epithelium of mouse (H&E, 50x).

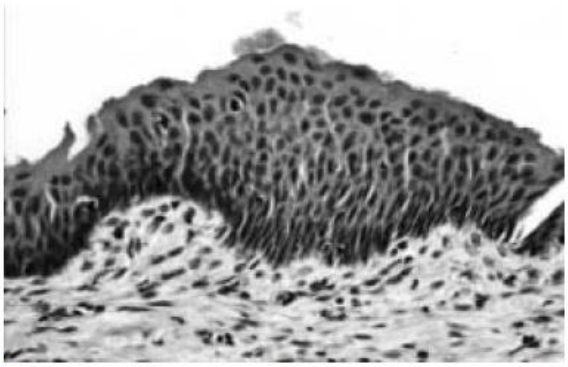

Fig. 4 - Dysplasia of the bladder epithelium of mouse (H&E, 100x).

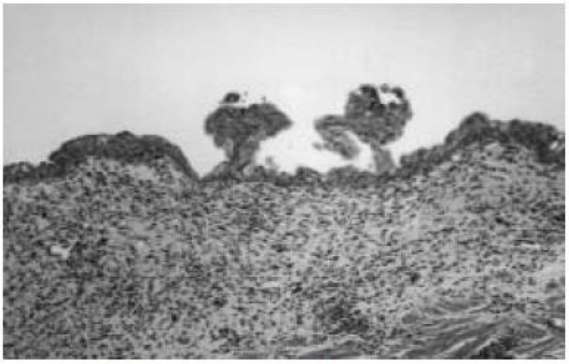

Fig. 5 - Papillary tumours of the bladder epithelium of mouse (H&E, 25x).

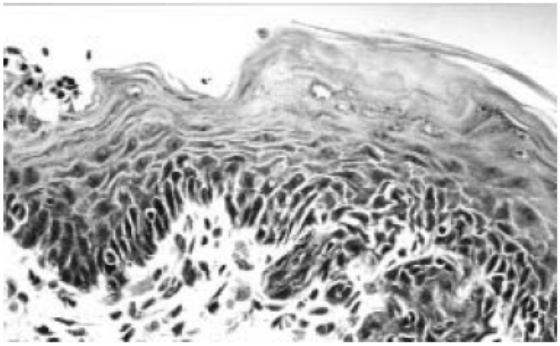

Fig. 6 - Squamous metaplasia of the bladder epithelium of mouse (H&E, 100x).

Discussion

Rodent models have proven to be useful in furthering our understanding of carcinogenesis. They allowed a detailed investigation of sequential events occurring during cancer evolution, metastatic growth, clinical diagnosis and evaluation of chemotherapeutic agents (26-29). The mouse has been suggested as an ideal model for studying urinary bladder carcinogenesis, because not only mouse urinary bladder spontaneous tumours are rare, but also other spontaneous non-neoplastic changes such as inflammation and epithelial hyperplasia occur infrequently (30). There are no known reports of parasitic infection in the mouse bladder. Chemical carcinogenesis in the mouse model simulates the major pathogenetic mechanism known in human bladder carcinogenesis (24,30). Bladder neoplasia in humans can be classified into two diseases, a low-grade papillary lesion that does not progress and metastasize, and a second, highly malignant neoplastic process that progresses through dysplasia, carcinoma *in situ* and invasion. In the mouse bladder carcinogenesis proceeds through a process of flat, nonpapillary dysplasia, carcinoma *in situ*, and invasive carcinoma (31). In the BBN mouse model, not all mice strains develop bladder tumours even at very high doses (24). Some nitroso compounds exhibit carcinogenic activity toward the urinary bladder, but BBN seems to affect only the urinary bladder in mice and rats (32). However, there appear to be distinct differences in induced bladder tumours by BBN between rats and mice. This species' variations in bladder cancer induction is closely related to the extent of urinary excretion of N-Butyl-N-(3-carboxypropil)nitrosamine, the major urinary metabolite of BBN (33).

In this study, we have confirmed that 0.05% BBN administration induces the development of simple hyperplasia, dysplasia, papillary tumors and squamous cell metaplasia. Our principal aim was to analyse DNA content of BBN induced urothelium lesions. The identification of prospective malignancy in borderline lesions and dysplasia by histopatology alone is often insufficient for the early and definitive detection of malignancy. These diagnoses result from the insufficiency of morphological criteria to clearly discriminate between benign and malignant cells if they look simi-

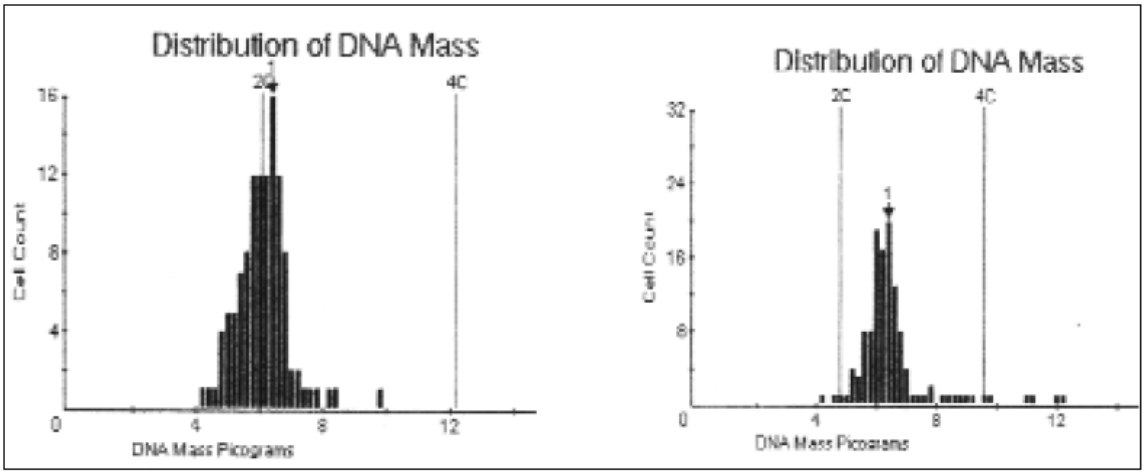

Fig. 7 - Image analysis DNA histogram of a hyperplasia showing a DNA diploid cell population.

Fig. 8 - Image analysis DNA histogram of a dysplasia showing a DNA aneuploid cell population.

Table II - Correlation between DNA ploidy, lesions and BBN exposition.

BBN weeks	DNA ploidy	Normal urothelium n (%)	Hyperplasia n (%)	Dysplasia n (%)	Papillary tumour n (%)	Squamous cell metaplasia n (%)
4	Diploid	5(100%)	7(77.8%)	5(55.5%)	0	0
	Aneuploid	0	2(22.2%)	4(44.5%)	1(100%)	0
8	Diploid	5(100%)	5(62.5%)	2(25%)	0	0
	Aneuploid	0	3(37.5%)	6(75%)	0	0
12	Diploid	5(100%)	2(28.5%)	1(14.3%)	0	2(100%)
	Aneuploid	0	5(71.5%)	6(85.7%)	1(100%)	0

Table III - DNA ploidy in hyperplasia and dysplasia lesions.

Lesion	Diploid	Aneuploid	p value
Hyperplasia (n=24)	14(58.3%)	10(41.7%)	0.041
Dysplasia (n=24)	7(29.13%)	17(70.84%)	

lar. In dysplasia it is not evident if the cells are non-specifically altered or if they have been already transformed into malignant cells (34).

We used ICM to measure cell DNA content. This DNA analysis is time consuming, however results obtained with our material offers the possibility of simultaneous analysis of different types of histopathological lesions in the same sample, an advantage when compared to flow cytometry. This is due to the microscopic screening of samples for the detection of minor abnormal population of cells. Used in conjunction with light microscopy, measurements of DNA ploidy can be important in clinical practice (35).

DNA aneuploidy is equivalent to chromosomal aneuploidy. Detection of chromosomal or DNA aneuploidy means presence of malignancy. However, lacking evidence of DNA aneuploidy does not exclude neoplasia or malignancy. The unequivocal evidence of DNA aneuploidy by evaluation of DNA histograms may become obligatory in precancerous lesions (36). Aneuploid cells are more error-prone than normal cells hence aneuploid per se has significance. It is of clear interest that under the same cell proliferative stimulation condition, DNA content in hyperplasia, dysplasia and papillary tumours gradually increased. The finding that some of the hyperplastic urinary bladder samples were aneuploid suggests that DNA aneuploidy may be associated with premalignant lesions of the urinary bladder. This is in line with the concept that lesions committed to cancer development have a lower potential to reversibility than reactive proliferative lesions, this is an important factor for the future occurrence of bladder carcinoma (37,38). Our results suggest that DNA content may be used to characterize the same type of lesion at different periods of BBN exposition. After 4, 8 and 12 weeks of BBN exposition we observed a gradual increase in the frequency of aneuploidy in hyperplasia and dysplasia lesions, however the percentage of bladder lesions were similar after 4, 8 and 12 weeks of BBN exposition, the carcinogenic effect of BBN is not seen by the morphology of lesions but by the increase in aneuploidy frequency. The detection of aneuploidy in hyperplasias and dysplasias after 4 weeks of BBN exposition reveals the sensibility of this technique to evaluate DNA content and this model remains actual to study bladder carcinogenesis. We sought to learn more about the histological behaviour of hyperplasia and dysplasia and their prospective significance in the development of bladder carcinoma, and to draw from such animal experiments conclusions regarding carcinogenesis in humans. It, thus, appears that aneuploidy reflects a disturbance of the genome correlated to the presence of malignant transformation and that aneuploidy can be used to discriminate between nonspecific, non-preneoplastic from preneoplastic lesions (15). In addition, nuclear DNA content can, at best, only be a marker for malignat potential. The clearly aneuploid and variable DNA cytophotometric pattern in aneuploid lesions may reflect a high degree of genomic instability in such cell population

an instability which in itself may lead to a rapid generation of new phenotypes that may be a prerequisite for a rapid progression of the neoplasic disease (15,20,39). While it is well documented that genomic instability is involved in tumour progression, only recently genomic instability has been seriously considered as a possible initiating event in oncogenesis (40). DNA measurement may be used as an important objective predictor, complementary to traditional morphologic parameters. Further studies on mice should provide information on the mechanisms of aneuploidy development. Although mouse models can never truly mimic the human, these results could elevate the chance of transformation of preneoplastic into malignant lesions.

Abbreviations: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; ICM, image cytometry analysis, W, weeks; DNA, deoxyribonucleic acid.

Acknowledgements. The authors would like to thank Drª Inês Godinho for the English version and paper review.

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Received: March 13, 2005

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Artigo #5: DNA cytometry and kinetics of rat urothelial lesions during chemical carcinogenesis.

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Oncol Rep. 2009; 21: 247-252.

ONCOLOGY REPORTS 21: 247-252, 2009

DNA cytometry and kinetics of rat urothelial lesions during chemical carcinogenesis

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Received July 30, 2008; Accepted September 12, 2008

DOI: 10.3892/or_00000215

Abstract. The aims of this study were to evaluate the DNA content of chemically-induced rat urothelial lesions and their relationship to the proliferation index and histological patterns. Sixty female Fisher 344 rats were divided randomly into six groups, four groups were exposed to N-butyl-N-(4-hydroxybutyl) nitrosamine for a period of 10 and 20 weeks, and two groups of ten rats were used as control animals. Paraffin sections were Feulgen stained and analyzed using DNA image cytometry analysis; histograms were classified as either diploid or aneuploid. Ki-67 immunoreactivity was determined by means of the streptavidin-biotin-complex immunoperoxidase method. All normal urothelium from the control groups were found to have diploid DNA content. The same histogram pattern was found in the simple hyperplasia group. As regards the other histological lesions, the frequency of the aneuploidy varied depending on the lesion type: 20% of aneuploidy were nodular hyperplasia, 32% of aneuploidy were dysplasias, 25% of aneuploidy were papilloma, 44% of aneuploidy were papillary neoplasm of low malignant potential, 22% of aneuploidy were low-grade papillary carcinoma, 100% of aneuploidy were high-grade papillary carcinoma and 100% of the aneuploidy were invasive carcinoma. Our results revealed the existence of a statistically significant relationship between DNA ploidy and histological pattern lesions (r=0.3, p<0.023). The Ki-67 proliferation index was significantly higher in aneuploid lesions than in diploid (r=0.56, p=0.01). There was also a statistically significant difference in the Ki-67 proliferation index in relation to the histopathological pattern (r=0.751, p<0.01). DNA content was associated with the Ki-67 proliferation index and histopathological grade. DNA content and proliferation index have critical roles to play during urothelial carcinogenesis.

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Key words: Rat, DNA image cytometry, urothelial lesions, proliferation index

Introduction

Animal models for studying bladder carcinogenesis have reliably demonstrated that the urothelium is a good choice for studying the early alterations that occur during the initial steps of the cancerization process. The main morphological stages have been identified, progressing from simple hyperplasia to invasive carcinoma, and are similar to those observed in man (1-3). For this reason, these models are a valuable tool for understanding the genetic, epigenetic and environmental factors that influence cancer progression and behaviour. Since bladder tumours exhibit heterogeneous behaviour patterns, the study of this disease cannot be based only on anatomopathological criteria. DNA ploidy provide valuable information on the biological behaviour of preneoplastic and neoplasic lesions (4,5). DNA image cytometry is a technique that enables the densitometric analysis of nuclear DNA of cells and therefore the individual evaluation of DNA ploidy. As a complementary method for this kind of individual molecular markers, gross genomic damage measurements in the form of aberrant DNA content could prove to be a valuable method in the identification of premalignant events and markers to evaluate the different prognosis observed from malignant lesions (4,6,7).

A tumour's proliferative rate has been considered to help predict its subsequent evolution (8,9). Ki-67 is a proliferation associated nuclear antigen that is expressed in replicating cells throughout all phases of the cell cycle (G1, S, G2, M) but is not expressed in quiescent cells (G0) (10). The immunohistochemical expression of Ki-67 has been used in tumour proliferation assessment for many malignancies such as gallbladder, lung, prostate, bladder and breast (11-14). It is also considered to be an acceptable proliferative marker in

rodent urothelial carcinogenesis (15,16). The relationship between DNA content and Ki-67 expression in chemically-induced urothelial lesions remains to be clarified. To the best of our knowledge, there are as yet no reports published on the relationship between DNA content and proliferation index during rat urothelial chemical carcinogenesis. This study uses image cytometry analysis to evaluate the DNA content of rat urothelial lesions that are chemically induced by N-butyl-N-(4-hydroxybutyl) nitrosamine. It also aims to assess the kinetic features of the same lesions by determining their proliferation index and correlating these values with histological patterns.

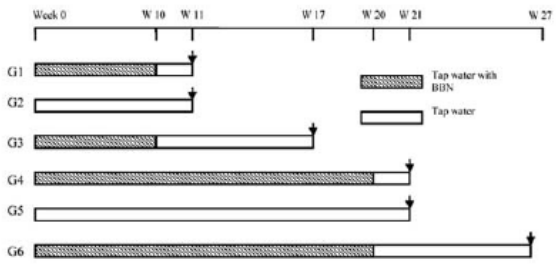

Figure 1. Experimental design (arrow indicates time of death; W-week).

Materials and methods

The Portuguese General Veterinary Direction in accordance with the current regulations and standards currently in place in the European Community, approved the facilities where the animals were kept and the experimental procedures. Sixty, five week-old, female Fisher 344 rats were obtained from Harlan (Amsterdam, The Netherlands). The rats were randomly housed in plastic cages, with hard wood chips used for bedding (cages were cleaned twice a week). They were kept under controlled temperature conditions (23±2˚C), light-dark periods of 12 h and with free access to water and a commercially available diet (Harlan Teklad, Glogal Diet). After a one-week acclimatization period, four groups of ten animals were given drinking water containing 0.05% BBN (Tokyo Kasei Kogyo Co., Tokyo Japan) for a period of 10 (groups 1 and 3) and 20 weeks (groups 4 and 6). Twenty animals were used as negative controls; they drank water without BBN for 10 (group 2) and 20 (group 5) weeks. Rats were sacrificed and necropsies were carried out as detailed in Fig. 1. During the course of the experiment, a daily health check was performed and body weights were measured once per week.

For histological examination purposes, the bladders were filled with 10% buffered formalin, the urethra was ligated with silk thread and the whole bladder was fixed and 12 h later cut sagittaly. The urinary bladders were embedded in paraffin. Sections of 2 μm were cut and stained with haematoxylin and eosin. All sections were reviewed by two researchers and the urothelial lesions classified according to the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (17). Urothelial lesions were categorized as either: simple hyperplasia, nodular hyperplasia, dysplasia, papilloma, papillary neoplasm of low malignant potential, low-grade papillary carcinoma, high-grade papillary carcinoma, invasive carcinoma, spinocellular carcinoma or squamous metaplasia.

The nuclear DNA content of preneoplastic and neoplastic lesions was measured using the CAS 200 Image Analysis System (Cell Analysis Systems, Elmhurst, III, Becton-Dickinson®). Urinary bladder sections from paraffin-embedded blocks were cut at 6 μm before being deparaffinized and rehydrated. The slides were then Feulgen-stained with the CAS DNA staining kit (Cell Analysis Systems, Elmhurst, III) as previously described (18). The Feulgen reaction produced a blue staining of nuclear DNA which reflects the stain's stoichiometric binding to the DNA. At least 100 complete, non-overlapping, and focused nuclei in every case and 20-30 normal lymphocytes (used as internal reference diploid cells) were measured. The resultant DNA histograms were analysed and classified as diploid and aneuploid using a previously described methods (19). The 5c exceeding rate (5cER) defined as the percentage of tumour nuclei with DNA content above 5n, was also evaluated.

Immunohistochemistry was performed using the streptavidin-biotin-complex immunoperoxidase method as

Table I. Incidence of urothelial lesions in female Fisher 344 rats exposed to BBN and control groups. No. of animals (%).

Group (n)	Normal urothelium	Simple hyperplasia	Nodular hyperplasia	Dysplasia	Papilloma	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma	Invasive carcinoma	Spinocellular carcinoma	Squamous metaplasia
1 (10)	0	10 (100)	4 (40)	10 (100)	3 (30)	4 (33.3)	1 (10)	0	0	0	4 (40)
2 (10)	10 (100)	0	0	0	0	0	0	0	0	0	0
3 (10)	0	8 (80)	9 (90)	10 (100)	5 (50)	5 (50)	6 (60)	1 (10)	0	0	3 (30)
4 (10)	0	3 (30)	9 (90)	10 (100)	5 (50)	5 (50)	7 (70)	2 (20)	2 (20)	1 (10)	6 (60)
5 (10)	10 (100)	0	0	0	0	0	0	0	0	0	0
6 (10)	0	0	9 (90)	10 (100)	3 (30)	6 (60)	10 (100)	9 (90)	1 (10)	0	5 (50)

PNLMP, papillary neoplasm of low malignant potential.

previously described (20). Immunohistochemical staining for Ki-67 (1:20, M7248, Dako®) were performed to study proliferation index. For each lesion, cells showing positive staining within several microscopic fields with the highest immunoreactivity were counted at a higher magnification (x400), using a 10x10 grid. The Ki-67 labelling index (LI) was calculated as the percentage of positive nuclei divided by the total number of cells examined. At least 500 cells per lesion were examined. DNA content and proliferation analysis were performed on the same areas.

Statistical analysis of the data was carried out using the Statistical Package for Social Sciences for Windows (SPSS, Inc., Chicago, IL). Data were expressed as the mean ± standard deviation. The numeric parameters were tested by ANOVA or Kruskal Wallis tests. Histological grade was evaluated by χ^2 or Fisher tests (two-sided). The Spearman correlation was used to evaluate the association of ploidy with histological pattern. P-values <0.05 were considered statistically significant.

Results

There were no significant differences in the body weight gained during the course of the experiment between the BBN-treated and control rats. No deaths attributable to the BBN treatment were observed. No macroscopic or microscopic changes were seen in the liver, lungs, kidneys and gastrointestinal tract. Macroscopically, the urinary bladder was normal in all rats belonging to the control group. Urinary bladders from animals treated for 10 weeks with BBN were apparently normal. Greyish-white urinary bladder masses varying in size between 0.1-7 mm (size at largest diameter) were observed in those groups exposed to BBN for 20 weeks. The surfaces of the lesions were irregular with necrotic and, haemorrhagic areas as well as focal ulcerations. The lesions were distributed randomly throughout the entire urinary bladder. The majority of those lesions were pedunculate. Stone formation in the urinary bladder was not observed in any rat. No histopathological changes in urothelial cells were observed in the control group. The incidence of histopathological lesions in each group is shown in Table I. Simple and nodular hyperplasia, dysplasia, papilloma, papillary neoplasm of low malignant potential, low-grade papillary carcinomas were observed in all the bladders of all the BBN-treated rats. High-grade papillary carcinomas and invasive carcinomas were only observed in animals euthanized after the 7th week.

Adequate DNA histograms were obtained for all the urothelial lesions examined by image cytometry analysis. ICM DNA analysis was successfully performed on 92 lesions and 20 normal urothelium. All the DNA histograms of the normal urothelium from control groups were DNA-diploid. DNA-aneuploid histograms were found only in urothelial lesions. The DNA content results are summarized in Table II.

Despite the period of observation and the BBN treatment, the frequency of the DNA aneuploid pattern increased with the degree of tissue transformation and this variation was statistically significant ($p<0.05$).

Urothelium from the control group were negative for Ki-67 expression, and in the urothelial lesions Ki-67 immuno-

Table II. DNA content results according to the histological pattern and animal group.

Histological pattern	n	DNA content	
		Diploid n (%)	Aneuploid n (%)
Group 1			
Simple hyperplasia	5	5 (100)	0
Nodular hyperplasia	1	1 (100)	0
Dysplasia	7	3(42.8)	4 (57.2)
Papillary neoplasm of low malignant potential	2	1 (50)	1 (50)
Squamous metaplasia	1	1 (100)	0
Group 2			
Normal urothelium	10	10 (100)	0
Group 3			
Simple hyperplasia	3	3 (100)	0
Dysplasia	6	5(83.3)	1 (16.7)
Papillary neoplasm of low malignant potential	5	4 (80)	1 (10)
Low-grade papillary carcinoma	4	3 (75)	1 (25)
High-grade papillary carcinoma	1	0	1 (100)
Group 4			
Nodular hyperplasia	3	2(66.7)	1 (33.3)
Dysplasia	7	5(71.4)	2 (28.6)
Papilloma	4	3 (75)	1 (25)
Papillary neoplasm of low malignant potential	5	2 (40)	3 (60)
Low-grade papillary carcinoma	4	3 (75)	1 (25)
High-grade papillary carcinoma	2	0	2 (100)
Invasive carcinoma	2	0	2 (100)
Squamous cell carcinoma	1	1 (100)	0
Squamous metaplasia	1	0	1 (100)
Group 5			
Normal urothelium	10	10 (100)	0
Group 6			
Nodular hyperplasia	1	1 (100)	0
Dysplasia	5	4 (80)	1 (20)
Papillary neoplasm of low malignant potential	4	2 (50)	2 (50)
Low-grade papillary carcinoma	10	8 (80)	2 (20)
High-grade papillary carcinoma	5	0	5 (100)
Invasive carcinoma	1	0	1 (100)
Squamous metaplasia	2	2 (100)	0

Table III. Proliferation index in different urothelial lesions and experimental groups.

Group (n)	Simple hyperplasia	Dysplasia	Papilloma	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma
1	6.6±1.5	15.5±1.3	-	-	-	-
3	19.5±7.7	20±2.8	-	25.5±4.9	29.3±0.6	-
4	-	20.8±4.6	17.6±5.8[a]	24.4±3.1	29±6.8	29±1.41
6	-	17.3±4.2[b]	-	22.3±4.7	24.8±4.5	32±1.58

PNLMP, papillary neoplasm of low malignant potential. [a]Statistically different from low-grade papillary carcinoma (p=0.026). [b]Statistically different from high-grade papillary carcinoma (p=0.003).

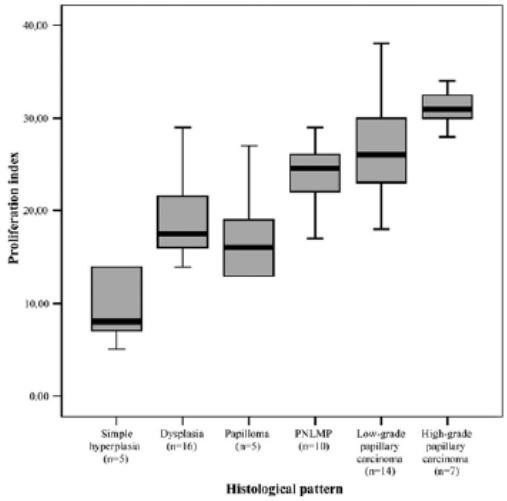

Figure 2. Proliferation index in different histological patterns.

Table IV. Significance of proliferation index between different histological patterns.

	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma
Simple hyperplasia	SD	SD	SD
Dysplasia	-	SD	SD
Papilloma	-	SD	SD

SD, statistically different (p<0.05).

staining was evident as diffuse or dotlike nuclear and nucleolar staining. Ki-67 expression was evaluated in 57 lesions and the mean values of the proliferation index for each lesion in each group evaluated are summarized in Table III.

When the mean values of the proliferation index for the various lesions were analyzed, a tendency to increase Ki-67 expression was observed in lesions with a high grade of malignancy (Fig. 2). Variance analysis using the Bonferroni test showed significant differences (p<0.05) between the mean value of the high-grade malignancy when compared with other lesions (Table IV).

To clarify the biological significance of Ki-67 during urothelial carcinogenesis, a fixed linear model was fitted, which had the histological pattern and ploidy status as the independent variables. Both factors had a statistically significant influence on the proliferation index (p<0.001). A significant correlation was found between DNA ploidy and histological pattern (r=0.3, p<0.02). Correlation between proliferation index and DNA ploidy was r=0.563 and p<0.01. Correlation between proliferation index and histological pattern was r=0.751 and p<0.01 (Fig. 3).

Discussion

During the transformation from a normal to malignant state a number of DNA alterations occur. These according to the concept of genetic instability, enable a stepwise evolutionary selection of tumour cell populations with the biological characteristics of tumour progression (21). Few studies have been reported on the correlation between tumour DNA ploidy and proliferation index in urothelial preneoplastic and neoplasic lesions (22,23). As regards rat urothelial lesions, no other study on this subject can yet be found in the available literature. The results of our research revealed that simple hyperplasia were 100% diploid and that a progressive increase in aneuploid frequency was observed as the malignant potential of the lesion increased, until there were 100% of aneuploidy in invasive carcinoma. Our findings are in general agreement with those previously reported by us on chemically-induced preneoplastic lesions in mice (18). Urothelial dysplasia has been considered a putative precursor of carcinoma *in situ* (CIS), and CIS of invasive urothelial carcinoma (17). DNA aneuploidy of dysplasia reflects the precancerous nature of this histological lesion, since the presence of aneuploidy provides unequivocal proof of malignancy. The detection of this neoplastic transformation prior to observing the morphological evidence of the neoplasia, enhances the usefulness of DNA content evaluation in preneoplastic lesions. Evaluating DNA content via image cytometry analysis makes it possible to distinguish between neoplastic cells and void non-tumour cells, as image

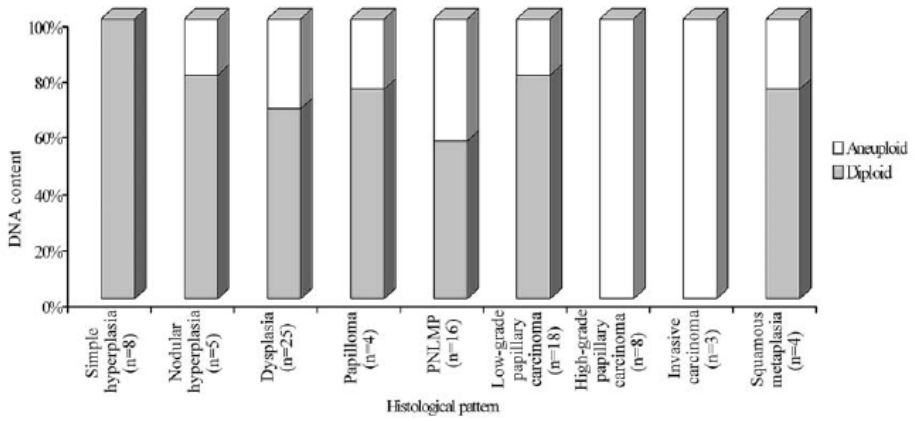

Figure 3. DNA ploidy observed in the different histological patterns analysed.

cytometry analysis enables the simultaneous analysis of different types of histopathological lesions within the same sample (18). In the present study, a strong correlation was established between DNA-ploidy status and histopathological grading. Nevertheless, we found that lesions of the same histological type might have different DNA content depending on their exposure to BBN. These results indicate an increased specificity, of the positive benefits of using DNA cytometry, and confirm this method's suitability for studying urothelial carcinogenesis. DNA content analysis has been reported as diagnostically useless in differentiating benign from malignant conditions (24,25). The data presented here show that the evaluation of cell proliferation using Ki-67 is closely related to the malignancy grades determined by histopathological methods. In common with the results obtained by other researchers (26), the data in this study showed a statistically significant difference between the histopathological grade and the Ki-67 expression in preneoplastic and neoplastic cells. Lesions with a high grade of malignancy showed a greater percentage of Ki-67 positive cells than preneoplastic lesions or low-grade papillary carcinomas. These findings suggest that the evaluation of Ki-67 expression in urothelial lesions can improve our knowledge of bladder cancer development. As regards DNA content, this study demonstrated that DNA content is correlated with tumour proliferation; the Ki-67 proliferation index was significantly higher in aneuploid lesions than in diploid lesions. Other researchers studied the relationship between cellular proliferation and DNA content in adrenocortical proliferative lesions and oesophagus neoplasic conditions and concluded that both factors were related (27,28).

Collectively taken, our data indicate that DNA ploidy and tumour cell proliferative activity, as determined by the expression of Ki-67 nuclear antigen are correlated with the various histological patterns observed during the course of the urothelial malignant transformation process. If the conclusions drawn by our study were to be confirmed by other studies on urothelial carcinoma in man, then DNA ploidy status and Ki-67 taken together may help us to better understand urothelial carcinogenesis.

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Artigo #6: DNA content analysis, expression of Ki-67 and p53 in rat urothelial lesions induced by N-butyl-N-(4-hydroxybutyl) nitrosamine and treated with mitomycin C and bacillus Calmette-Guérin.

Oliveira PA, Palmeira C, Colaço A, de la Cruz LF, Lopes C.

Anticancer Res. 2006; 26: 2995-3004.

ANTICANCER RESEARCH *26*: 2995-3004 (2006)

DNA Content Analysis, Expression of Ki-67 and p53 in Rat Urothelial Lesions Induced by *N*-Butyl-*N*-(4-Hydroxybutyl) Nitrosamine and Treated with Mitomycin C and Bacillus Calmette-Guérin

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Abstract. *N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced urothelial carcinogenesis is a useful model for studying urothelial carcinogenesis. Here, the DNA content and the expression of Ki-67 and p53 in urothelial lesions induced by BBN and treated with mitomycin C (MMC) and Bacillus Calmette-Guérin (BCG) were investigated. Female Fisher 344 rats were distributed into five groups treated with 0.05% BBN in their drinking water for 20 weeks. Ten animals were used as negative control. Intravesical instillations were performed with MMC, BCG and physiological saline solution (PSS), once per week, for 6 weeks. The animals were sacrificed 1 week after the last intravesical instillation. DNA ploidy analysis was carried out by static cytometry. Ki-67 and p53 were analysed immunohistochemically in paraffin-embedded tissue. The incidence of lesions developed in rats with PSS was greater than in rats instilled with MMC and BCG. The incidence of aneuploidy was lower in tumours treated with MMC and BCG. Low- and high-grade papillary carcinoma treated with MMC and BCG showed a decrease in labelling index and an increase of apoptotic index. The proliferative index was correlated with the apoptotic index ($r=0.438$, $p<0.01$). Significant correlations were also found between the proliferative index and lesion, and the apoptotic index and lesion ($r=0.425$, $p<0.01$ and $r=0.275$, $p<0.01$), respectively. A significant correlation was found between ploidy and the apoptotic index ($r=0.245$, $p<0.05$). Our results provide information on the biological behaviour of chemically-induced bladder tumours treated with MMC and BCG.*

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Key Words: DNA content, Ki-67, p53, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine, rat, urothelial carcinogenesis.

Bladder cancer is the ninth most common cancer worldwide (1), but is superficial at presentation in 70 to 80% of patients. Due to the excellent prognosis, initial treatment is usually by transurethral resection (2). However, further treatment is necessary either to eradicate residual malignancy or to lessen the likelihood of recurrence and progression (3). Intravesical chemotherapy with mitomycin C (MMC) and intravesical immunotherapy with Bacillus Calmette-Guérin (BCG) have been used intravesically to treat bladder cancer (3, 4).

Rodent carcinogenesis models featuring the production of urinary bladder carcinomas by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) are well established (5, 6). BBN is a genotoxic compound with potent and selective organotropic carcinogenic activity, inducing the development of neoplastic lesions in the urothelium of several animals (7-9). This substance, administered in drinking water to rats, induces urothelial tumours that morphologically resemble their human counterparts (10).

In recent years, many techniques have been used to predict the behaviour of urothelial tumours. The combination of DNA image cytometry with immunohistochemistry could lead increased knowledge of the efficacy of BCG and MMC in the treatment of chemically-induced bladder cancer. DNA image cytometry measurement of DNA ploidy has provided useful information regarding the biological behaviour of a vast range of human and animal neoplastic and preneoplastic lesions (11, 12). Different authors have investigated the influence of distinct antigens in bladder cancer (13). One of

the most common markers used is the Ki-67 labelling index. Ki-67 is a nuclear protein present during the G1-, S-, G2- and M-phases of cycling cells (14). Several studies have consistently shown that the labelling index, measured by Ki-67 immunoexpression, correlates with the growth of many tumours, including bladder cancer (15). The p53 tumour suppressor gene encodes for a protein that plays an important role in cell cycle regulation and apoptosis mechanisms (16). Gene mutations in this tumour suppressor gene result in the production of an altered protein, which does not function correctly or have a prolonged half-life (17). Immunohistochemical p53 detection is correlated with the amount of mutated p53 gene (16).

The aims of the current study were: a) to examine the effects of intravesical instillations of MMC and BCG on the development of bladder tumours induced in rats by BBN; b) to study the DNA content in lesions induced by BBN and treated with BCG and MMC; c) to characterise the expressions of Ki-67 and p53 in the urothelial tumours induced by BBN and treated with BCG and MMC.

Materials and Methods

Animals, diet, carcinogen and experimental design. The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation (Direcção Geral de Veterinária, Approval no. 520/000/000/2003). Sixty-seven female Fisher 344 rats were obtained at the age of 5 weeks from Harlan (Amsterdam, Netherlands). The rats were used in this study after a week of acclimatization. They were randomly housed 5 to 7 in a plastic cage, with hard wood chips for bedding and were maintained on a basal diet (Harlan Teklad, Global Diet). The room temperature and the relative humidity were controlled at 22±3˚C and 60±10%, respectively. Fluorescent lighting was provided in a 12-h light/dark cycle. BBN was purchased from Tokyo Kasey Kogyo (Japan) and administered in the drinking water, in light impermeable bottles, at a concentration of 0.05% to groups 1, 3, 4, 5 and 6. Group 2 served as the control without any chemical supplement. The experimental design is presented in Figure 1. The animals were observed daily. Body weights, as well as food consumption, were measured weekly. The drinking solution was changed twice per week and the volume that had been drunk was recorded. To evaluate histological changes induced in the urothelium by BBN treatment alone, the rats from group 1 were sacrificed 20 weeks after initial exposure. At the same time, animals from the control group were also sacrificed. The rats were sacrificed with 0.4% sodium pentobarbital (1 ml/Kg, intraperitoneal). The time of BBN exposure was based on previous studies (18). Group 6 was exposed to BBN for 20 weeks and was maintained with normal tap water until the end of the experiment. Groups 3, 4 and 5 were then maintained without any treatment for 1 week after which began intravesical instillations, once a week for 6 weeks, with MMC, BCG and physiological saline solution (PSS), respectively. Briefly, the animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). Before and 1 h after the instillation, micturition of the rats was induced by light abdominal massage so that the duration of exposure was constant. The

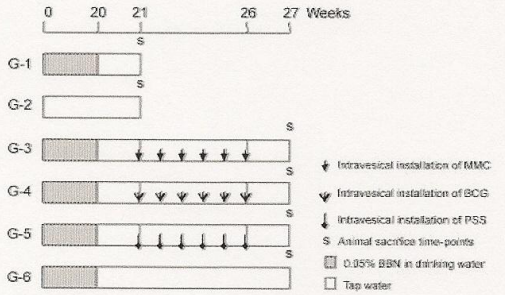

Figure 1. *Experimental design (G, group; MMC, mitomycin C; BCG, Bacillus Calmette-Guérin; PSS, physiological saline solution).*

bladder was catheterized *via* the urethra with an 18-gauge plastic intravenous cannula. All the experiments described herein were carried out under aseptic conditions. The rats were turned 90 degrees every 15 min to facilitate whole bladder exposure to the substances intravesically instilled. Body temperature was maintained with a homoeothermic bandage. After recovery from the anaesthesia, the rats were placed in individual standard cages for 12 h and were monitored daily for haematuria.

For intravesical immunotherapy, lyophilised Connaught BCG strain was kindly provided by INIBSA (Portugal), each vial containing 81 mg of lyophilised Connaught BCG with at least $1.8x10^8$ colony-forming units. Companhia Portuguesa de Higiene (Portugal) kindly provided the MMC (Mitomicina-C Kyowa). Both compounds were reconstituted with PSS, according to the manufacturer's instructions. Dosing schedules were based on those commonly used in clinical work; 300 μl of MMC solution (1 mg/1 ml) and 300 μl of BCG solution (81 mg/3 ml) (19, 20) were used.

All surviving animals were sacrificed 1 week after the last instillation by intraperitoneal administration of sodium pentobarbital. Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated *in situ* by injection of 10% phosphate-buffered formalin (1 ml), ligated around the neck to maintain proper distension and then were immersed in the same solution for 12 h. After fixation, the formalin was removed; the urinary bladder was weighed and cut into four strips and was routinely processed for haematoxylin and eosin staining.

Histopathological analysis. All sections were reviewed by two researchers and the urothelial lesions staged by the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (21). The urothelial lesions were categorized as simple hyperplasia, nodular hyperplasia, dysplasia, carcinoma *in situ* (CIS), papillary tumours, papillary neoplasm of low malignant potential, low-grade papillary carcinoma, high-grade papillary carcinoma, invasive carcinoma, spinocellular carcinoma or squamous metaplasia.

Quantification of DNA content. The ICM methods and the DNA histogram analysis used were previously described in detail (12) and are summarized here. For each lesion, the G0/G1-peak visually

Table I. *Average body weight, food and water intake for female Fisher 344 rats exposed to BBN followed by MMC, BCG and PSS intravesical instillation.*

Group	Initial body weight (g)	Final body weight (g)	Initial food intake (g/rat/day)	Final food intake (g/rat/day)	Initial water intake (g/rat/day)	Final water intake (g/rat/day)
1	130.57±5.09[a]	215.23±9.28	11.87	10.56	18.97	19.77
2	131.29±5.39	209.71±7.15	11.92	9.96	18.76	19.78
3	132.20±5.83	203.70±20.66	11.82	8.69	22.75	19.59
4	132.36±10.27	196.23±15.44*	11.87	8.80	21.16	18.92
5	130.17±8.48	188.33±16.70**	10.67	7.78	17.63	17.21
6	132.25±11.14	217.48±10.59	9.97	10.98	17.50	18.54

[a]Values are mean values ± SEM(standard errors of the mean).*,**Statistically different from group 6 ($p<0.05$).

identified, mean, standard deviation (SD) and the coefficient of variation (CV) values were calculated. The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of the mean DNA content of the urothelial G0/G1-peak divided by the mean DNA content of the resting diploid lymphocyte G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with values above 5 n. Lesions were considered aneuploid only if a separate G0/G1-peak was distinguishable on the histogram and if it differed from the reference lymphocyte population by more than 2 SD, (two standard deviations). A DNA diploid lesion showed a single distinct G0/G1-peak with a DI within 2 SD from the control lymphocytes and usually with less than 1% of 5cER.

Immunohistochemistry. The expression of markers was detected using the three-step streptavidin-biotin immunoperoxidase method. The mouse monoclonal antibody Ki-67 (1:20, M 7248, DakoCytomation) was used to identify proliferative activity. The p53 expression was performed using AB-1 monoclonal antibody (1:50; MS-104-PO, Neomarkers-Labvision). Tissue sections from paraffin-embedded tissue were deparaffinised in xylene, rehydrated through a down-graded alcohol series and washed with phosphate-buffered saline (PBS). To improve antigen detection, the sections were pre-treated in a microwave oven (700 W) for 20 min in a 10 mM citrate buffer pH 6.0 or EDTA buffer pH 8.0, for Ki-67 and p53, respectively. After cooling, the sections were immersed in 3% hydrogen peroxide (H_2O_2) and distilled water for 30 min to block endogenous peroxidase activity. Non-specific staining was eliminated by 30-min incubation with normal rabbit serum (X 0902, DakoCytomation). Excess normal serum was removed, replaced by the primary antibodies used and incubated overnight (4°C) in a humidified chamber. After washing the slides, the sections were incubated with a 1:400 dilution of biotin-labelled anti-mouse (E 0354, DakoCytomation) secondary antibody followed by the streptavidin-biotin-complex (TS-125-HR, Labvision Corporation) for 10 min at room temperature. Subsequently, the colour was developed with 3,3'-diaminobenzidine tetrahydrochloride with H_2O_2 in PBS buffer for 7 min. Slides were counterstained with Gill's haematoxylin, were dehydrated and mounted. Primary antibodies and biotinylated secondary antibodies were diluted in PBS. Negative controls were carried out by replacing the primary antibody with PBS. Paraffin sections from colon and breast cancer with known immunoreactivity to p53 and Ki-67 antigens, respectively, were used as positive controls.

For each case, positively-stained tumour cells within five microscopic fields with the highest immunoreactivity ("hot spot" areas) were counted at high magnification (400 X) using a 10x10 grid. The presence and location of the lesions were carefully controlled to guarantee that the Ki-67 and p53 sections contained the same lesion. The Ki-67 labelling index (LI) was calculated as the percentage of positive nuclei divided by the total number of cells examined. At least 1000 cells per lesion were examined. The reaction for p53 was considered positive when more than 10% of tumour cells exhibited strong diffuse immunostaining (22). The apoptotic index (AI) was calculated as the percentage of positive nuclei, based on an average of 1000 nuclei counted per lesion. All sections were blind analysed twice.

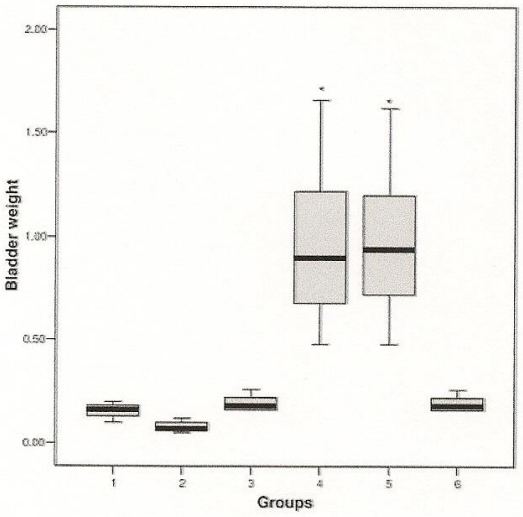

Figure 2. *Bladder weights in the different animal groups. *Statistically different from groups 3 and 6 ($p<0.05$).*

Analysis and statistics. A descriptive study was performed for all variables included. The statistical analysis was done using the SPSS 12.0 statistical package for Windows. The differences between the mean values of body weight, relative bladder weight, LI and AI were evaluated by analysis of variance (ANOVA) followed by the Bonferroni test. The differences in the incidence of lesions between

Table II. *Incidence of urothelial lesions in female Fisher 344 rats exposed to BBN and treated with MMC, BCG and PSS.*

Number of animals (%)												
Group (n)	Urothelium	Simple hyperplasia	Nodular hyperplasia	Dysplasia	CIS	Papilloma	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma	Invasive carcinoma	Spinocellular carcinoma	Squamous metaplasia
1 (12)	0	2(16.6)	10(87.3)	10(87.3)	0	4(33.3)	4(33.3)	10(83.3)	2(16.6)	2(16.6)	1(8,3)	6(50)
2 (10)	10(100)	0	0	0	0	0	0	0	0	0	0	0
3 (10)	0	1(10)	9(90)	10(100)	7(70)	0	3(30)	9(90)	8(80)	2(20)	0	5(50)
4 (8)	0	3(37.5)	8(100)	8(100)	0	1(12.5)	4(50)	7(87.5)	7(87.5)	2(25)	0	7(70)
5 (11)	0	1(9)	11(100)	11(100)	0	0	4(36.7)	11(100)	11(100)	5(45.4)	3(27.3)	10(91)
6 (10)	0	2(20)	10(100)	10(100)	0	3(30)	6(60)	10(100)	9(90)	1(10)	0	8(80)

PNLMP, papillary neoplasm of low malignant potential; CIS, carcinoma *in situ;*
BBN, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine; MMC, mitomycin C; BCG, Bacillus Calmette-Guérin; PSS, physiological saline solution.

the groups were assessed by Fisher's exact probability test or the Chi-square test. The Pearson correlation was used to evaluate the association of markers with each other, with the lesion and with ploidy. A $p<0.05$ value was accepted as statistically significant.

Results

General animal condition. Six rats from groups 3, 4 and 5 died during the instillation therapy and were therefore excluded from analysis. Animals surviving the 27 weeks were included in the effective numbers for histopathological examination. The initial and final average body weights, food and water intakes are summarised in Table I. The body weights of the rats in the groups treated with BCG and PSS (groups 4 and 5) were less than those of group 6. The mean food and water consumption were less in animals treated with PSS.

The relative bladder weights are summarized in Figure 2. The bladder weights in group 1 did not differ from those of group 2. The urinary bladder weights were significantly higher in groups 4 and 5 than those in groups 3 and 6.

Macroscopic and microscopic evaluation. Greyish-white urinary bladder masses, varying in size from less than 1 mm to masses that filled the entire lumen, were observed, those in groups 4 and 5 being the largest. The lesions were pedunculated with irregular surfaces and areas of necrosis, haemorrhage and focal ulcerations. The lesions were distributed randomly throughout the entire urinary bladder. Stone formation in the urinary bladder was not observed in any rat. No bladder mucosal lesions were seen that could be attributed to mechanical damage during intravesical instillation. The incidence of histopathological lesions in each group is shown in Table II. No histopathological changes in urothelial cells were observed in the control group. Simple and nodular hyperplasia and dysplasia were observed in all the bladders of the BBN-treated rats and the incidence of these lesions was approximately equal in the different groups. Seven animals in the group treated with MMC (group 3) had carcinoma *in situ* at the time of sacrifice. The incidence of tumours in rats treated with PSS was higher than in groups treated with MMC and BCG, although no statistical significance was observed. The incidence of both low- and high-grade papillary carcinoma decreased in groups 3 and 4, but there was no statistically significant difference between them and that in groups 5 and 6. The histopathological findings are shown in Figure 3. Histopathological inflammatory reaction, mainly lymphocytic aggregates, were observed in the submucosal layer of the groups 1, 3, 4, 5 and 6. Mast cells were present in all the animals from groups 1, 3, 4, 5 and 6. Populations of eosinophils and neutrophils were observed in the animals treated with BCG. No macroscopic or microscopic changes were seen in the liver, lung, kidney or gastrointestinal tract.

DNA content. ICM DNA analysis was successfully performed on 155 lesions and ten normal urothelia. The results for the DNA content obtained by image cytometry analysis are presented in Table III. The degree of aneuploidy in rats treated with PSS was greater than in those treated with MMC and BCG.

Two representative histograms obtained by ICM are shown in Figure 4. Despite the period of observation and the kind of treatment, the frequency of the DNA aneuploid pattern increased with the degree of tissue transformation ($p<0.01$) between high-grade papillary carcinoma when compared to normal urothelium, simple hyperplasia, nodular hyperplasia, dysplasia, papilloma, PNLMP and low-grade papillary carcinoma (Figure 5).

Immunohistochemistry. The normal urothelium in the control groups was uniformly negative for p53 and Ki-67

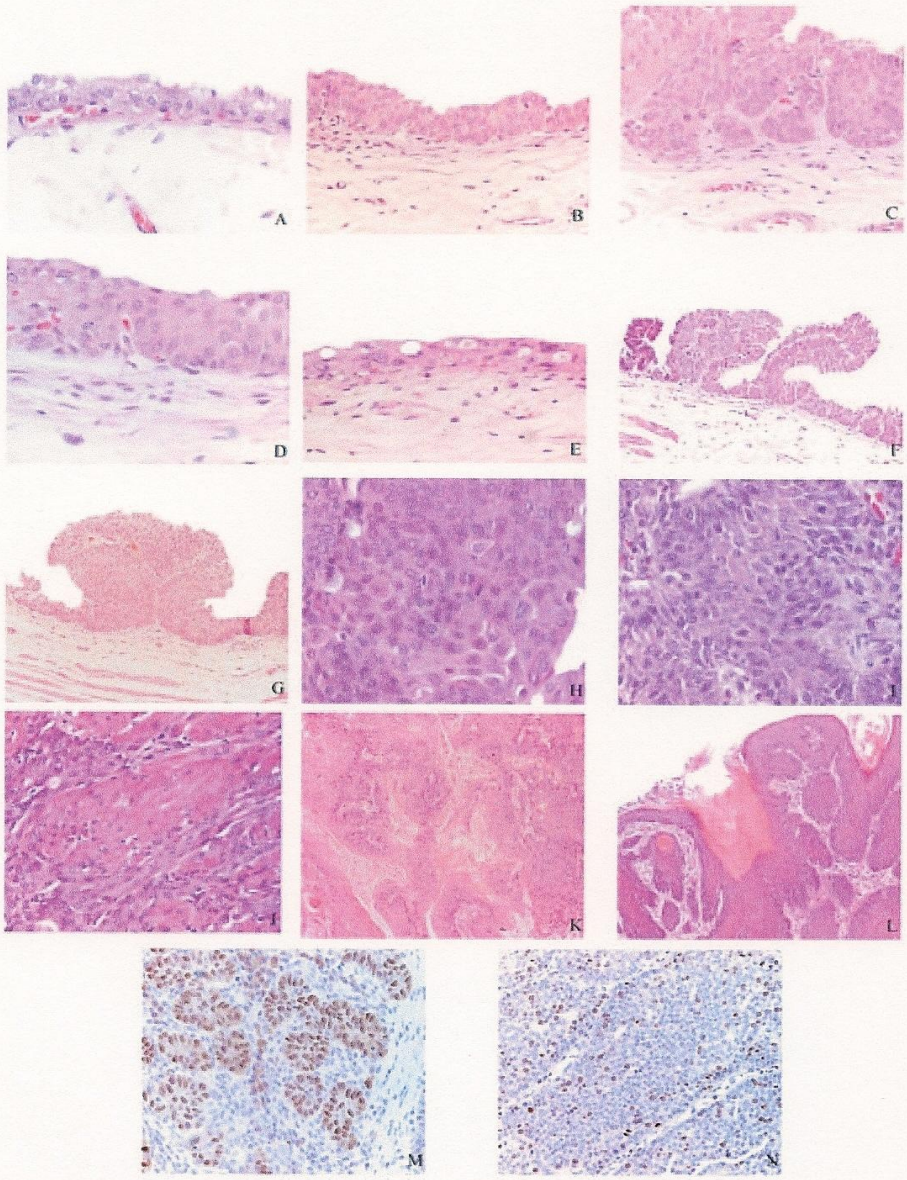

Figure 3. *Histopathological representation of lesions identified in rat urothelial carcinogenesis. A) Normal rat urothelium (Control group, H&E, 400x); B) simple hyperplasia (H&E, 400x); C) nodular hyperplasia (H&E, 400x); D) dysplasia (H&E, 600x); E) carcinoma in situ (H&E, 400x); F) papilloma (H&E, 200x); G) papillary neoplasms of low malignant potential (H&E, 200x) H) low-grade papillary carcinoma (H&E, 200x); I) high-grade papillary carcinoma (H&E, 600x); J) invasive carcinoma (H&E, 400x); K) spinocellular carcinoma (H&E, 200x); L) squamous metaplasia (H&E, 200x); M) p53 expression on low-grade papillary carcinoma (400x); N) Ki-67 expression on low-grade papillary carcinoma (200x).*

expressions. In terms of the Ki-67 and p53 immunoreactivity, 124 lesions showed positive immunoreactivity for both antibodies. Ki-67 immunostaining was evident as diffuse or dot-like nuclear and nucleolar staining (Figure 3N). p53 protein staining was confined to the nuclei (Figure 3M). The LI and AI were different within the various groups, showing the lowest values in group 1 and the highest in the group treated with PSS. The mean values of

Table III. *DNA content results regarding the histological pattern and animal group.*

Group	Histological pattern	Diploid n (%)	Aneuploid n (%)
	Simple hyperplasia (2)	2(100)	0
	Nodular hyperplasia (3)	2(66.7)	1(33.3)
	Dysplasia (6)	4(66.7)	2(33.3)
	Papilloma (4)	3(75)	1(25)
1	PNLMP (4)	2(50)	2(50)
	Low-grade papillary carcinoma (4)	3(75)	1(25)
	High-grade papillary carcinoma (2)	0	2(100)
	Spinocellular carcinoma (1)	1(100)	0
	Squamous metaplasia (2)	1(50)	1(50)
2	Normal urothelium (10)	10(100)	0
	Simple hyperplasia (1)	1(100)	0
	Nodular hyperplasia (2)	2(100)	0
	Dysplasia (3)	2(66.7)	1(33.3)
	CIS (7)	0	7(100)
3	PNLMP (1)	0	1(100)
	Low-grade papillary carcinoma (3)	2(66.7)	1(33.3)
	High-grade papillary carcinoma (6)	0	6(100)
	Invasive carcinoma (1)	0	1(100)
	Simple hyperplasia (3)	3(100)	0
	Nodular hyperplasia (4)	4(100)	0
	Dysplasia (8)	3(37.5)	5(62.5)
	Papilloma (1)	1(100)	0
4	PNLMP (2)	1(50)	1(50)
	Low-grade papillary carcinoma (8)	7(87.5)	1(12.5)
	High-grade papillary carcinoma (7)	0	7(100)
	Invasive carcinoma (1)	0	1(100)
	Squamous metaplasia (2)	2(100)	0
	Simple hyperplasia (1)	1(100)	0
	Nodular hyperplasia (6)	4(66.7)	2(33.3)
	Dysplasia (4)	1(25)	3(75)
5	PNLMP (2)	2(100)	0
	Low-grade papillary carcinoma (7)	4(57.1)	3(42.9)
	High-grade papillary carcinoma (7)	0	7(100)
	Invasive carcinoma (2)	0	2(100)
	Squamous metaplasia (5)	4(80)	1(20)
	Simple hyperplasia (1)	1(100)	0
	Nodular hyperplasia (4)	4(100)	0
	Dysplasia (3)	2(66.7)	1(33.3)
6	PNLMP (4)	2(50)	2(50)
	Low-grade papillary carcinoma (10)	8 (80)	2(20)
	High-grade papillary carcinoma (8)	0	8(100)
	Squamous metaplasia (3)	3(100)	0

PNLMP, papillary neoplasm of low malignant potential; CIS, carcinoma *in situ*.

LI and AI for each lesion evaluated are summarised in Table IV. The measurements (performed twice) of LI and AI were very well correlated ($r=0.95$, $p<0.001$).

The mean values of LI and AI for each lesion, regardless of the kind of treatment, are summarised in Figure 6. The highest values of LI and AI were observed in high-grade papillary tumours. Statistically, the proliferation index was correlated with AI; the Pearson correlation of these two markers was $r=0.438$ ($p<0.01$). A significant correlation was found between both LI and histological lesion ($r=0.452$, $p<0.01$) and AI and histological lesion ($r=0.275$, $p<0.01$). The correlation between ploidy and AI was $r=0.245$ and $p<0.05$.

Discussion

The histopathogenesis of BBN chemically-induced urothelial tumours has been well delineated (7, 8, 10). However, relatively little is known regarding the effects of MMC and BCG in the development of rat urothelial carcinogenesis. In this study, animals treated with PSS had the lowest body weight; they also showed a lower water and food consumption compared to the other groups. The bladder weights in groups 4 and 5 were significantly higher than in groups 1, 2, 3 and 6. The difference in bladder weight seems to be due to the different development of the bladder tumours.

The results of the present study indicated that both MMC and BCG exerted therapeutic effects against BBN-induced urinary tumorigenesis in rats. Inhibition of tumour growth occurred in animals treated with MMC and BCG compared to animals treated with PSS. These results are similar to those observed in C3H/He mice (23). The vehicle-treated animals (PSS) showed an increased incidence of low- and high-grade papillary carcinoma, invasive carcinoma and spinocellular carcinoma. Other researchers (18, 24) reported the promoter activity of PSS in urothelial tumours induced by BBN.

Intravesical therapy is an important adjuvant therapy after transurethral resection in patients with superficial bladder cancer and for the treatment of superficial bladder tumours (3, 4). MMC and BCG are often used as intravesical therapy, because these drugs cause little systemic toxicity and result in high concentrations in the bladder, in direct contact with tumours (10). There are several reports that intravesical instillation of MMC and BCG is effective for eradicating tumours and for inhibiting their recurrence in experimental models (25-27).

In the present work, the histological pattern classified as CIS was only found in the animal group treated with MMC. Szende *et al.* also identified CIS in female rats treated with MMC (28). Some authors reported a co-carcinogenic activity of MMC on urothelial carcinogenesis (29, 30). In a previous study by our group, the mitogenic and

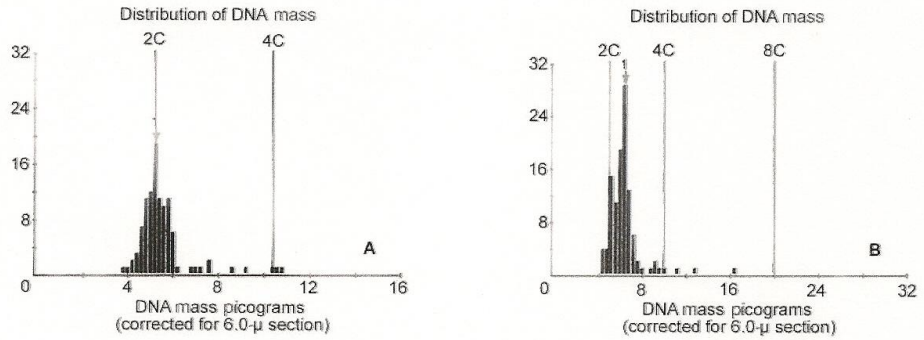

Figure 4. *(A) Image analysis DNA histogram showing a DNA diploid cell population. (B) Image analysis DNA histogram showing a DNA aneuploid cell population.*

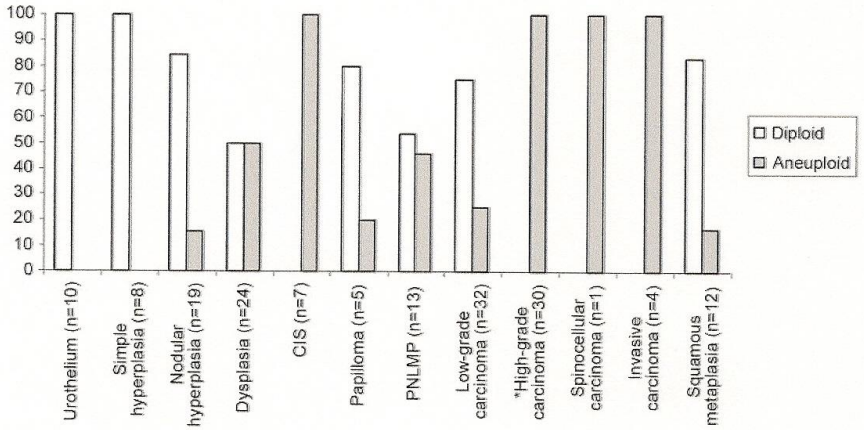

Figure 5. *DNA ploidy observed in the different histological patterns analysed. *Statistically different from normal urothelium, simple hyperplasia, nodular hyperplasia, dysplasia, papilloma, PNLMP and low-grade papillary carcinoma ($p<0.01$). CIS, carcinoma in situ; PNLMP, papillary neoplasm of low malignant potential.*

aneuploidogenic effects of MMC in rat normal urothelium were reported. More studies should be done for better understanding the biological mechanism of MMC therapy (submitted for publication by Oliveira *et al.*, 2005).

The DNA content of the urinary bladder lesions identified after intravesical instillation with MMC, BCG and PSS exhibited differences. The incidence of aneuploidy in rats treated with PSS was greater than in those treated with MMC or BCG. Lesions with morphologically identical cells sometimes have different biological behaviours. The use of ICM, by an individual cell evaluation, is a suitable method to detect the biological aggressiveness of a given lesion. Our results demonstrated that ICM provides more reliable results than classic histological methods. In this study, the evaluation of DNA ploidy in relation to the histopathological classification showed an aneuploid pattern with increasing frequency from simple hyperplasia to invasive carcinoma, as previously described in humans and mice (11, 12). During the progression of urothelial cell carcinoma, a selection of those cells with gains of genetic material seems to occur, conferring the DNA aneuploid status (31). Several preneoplastic lesions were aneuploid and these lesions frequently co-exist with bladder cancer and are hardly visible in humans during cytoscopy or may be concealed in the normal-appearing bladder mucosa. However, these lesions are of considerable importance in the recurrence and progression rates of bladder cancer. The use of ICM as a biomarker is biologically interesting since it reflects the

Table IV. *Association between LI and AI in the different urothelial lesions and experimental groups.*

Histological pattern	Group 1	Group 3	Group 4	Group 5	Group 6
Nodular hyperplasia					
LI	23.72±12.96[a]	21.71±0.51	13.59±5,82	23.71±5.24	20.10±11.31[b]
AI	28.50±0.70	35.50±3.53	30.50±2.88[b,c]	33.60±11.10	32.00±0.1[b]
Dysplasia					
LI	9.40±1.98	21.31±3.76	14.00±2.94[b]	22.15±6.43	23.50±0.70
AI	35.50±9.19	41.00±7.00	30.50±2.88[b,c]	36.00±4.96	45.00±1.41[e]
PNLMP					
LI	17.93±3.44	18.55±0.97	15.00±1.41	15.25±5.86[b]	21.33±3.21[b]
AI	23.33±3.05	33.00±1.41[f]	28.00±1.41[b,c]	21.50±0.70[b]	28.00±2.00[b]
Low-grade papillary carcinoma					
LI	17.58±3.46	29.41±4.95	20.80±6.57[e]	30.21±8.27	28.00±3.34
AI	35.66±5.89	39.66±5.77	42.28±3.19[g]	36.66±8.81	35.00±3.98[b]
High-grade papillary carcinoma					
LI	–	38.07±9.91	28.50±8.11	35.28±4.26	34.62±2.05
AI	–	45.60±6.42	50.00±4.85[d]	39.57±10.32	44.00±3.80
Invasive carcinoma					
LI	–	16.88±1.33	17.98±2.51	23.94±0.55	–
AI	–	26.83±2.30	27.50±2.12[b,c]	38.85±0.21[g]	–
Squamous metaplasia					
LI	24.92±9.74	32.11±4.08	31.05±2.76	31.79±8.71	32.40±5.82
AI	28.00±6.73	36.50±0.70	47.00±2.82	40.72±3.36	41.80±7.46

[a]values are mean values ± SEM (Standard errors of the mean); LI, labelling index; AI, apoptotic index; PNLMP, papillary neoplasm of low malignant potential; PSS, physiological saline solution. [b]Statistically different from high-grade papillary carcinoma. [c]Statistically different from high-grade papillary carcinoma. [d]Statistically different from all other lesions. [e]Statistically different from PNLMP. [f]Statistically different from animals treated with PSS. [g]Statistically different from animals treated with BCG.

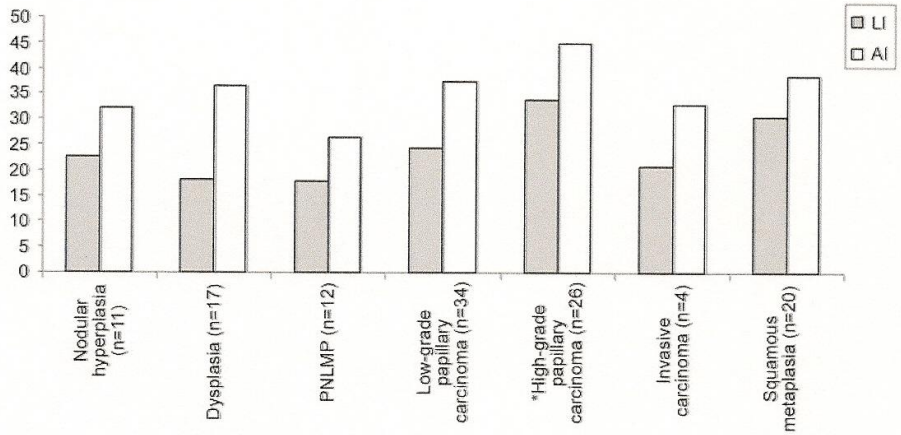

Figure 6. *Mean values of LI (labelling index) and AI (apoptotic index) in each lesion evaluated. *LI and AI statistically different from preneoplastic lesions and other tumours except squamous metaplasia ($p<0.05$). PNLMP-, papillary neoplasm of low malignant potential.*

phenotypic expression of malignancy, is reproducible and is easily applicable in routine practice (17, 32, 33).

It is interesting to note that exposure of the urothelium to MMC and BCG affected the expression of tumour markers. The immunoexpressions of Ki-67 and p53 differed between groups treated with BCG, MMC and PSS. In general, rats treated with MMC and BCG showed the lowest LI values compared to those instilled with the vehicle, suggesting that these compounds decrease cell proliferation. After MMC and BCG intravesical therapy, AI increased particularly in the high-grade papillary carcinoma. Thus, MMC and BCG intravesical therapy may act throughout the induction of

apoptosis and high-grade papillary carcinoma cells may be much more sensitive to this therapy.

We observed a statistically significant concomitant increase in Ki-67 and p53 expression patterns with the degree of tissue transformation. Ki-67 expression was studied in tumours of different organs and was shown to correlate with the histopathological pattern (34). p53 nuclear accumulation in bladder carcinomas has been reported to be correlated with the labelling index (35, 36). The correlation between apoptosis and proliferation in tumour tissues is important, since the rate of tumour cell accumulation is not only dependent on the rate of cell death, but also on the rate of tumour cell proliferation (22).

In conclusion, our results revealed that repeated instillation of MMC and BCG decreased tumour incidence in the urothelium of rats exposed to BBN. These lesions presented a low LI, a high AI and the incidence of DNA aneuploidy decreased. On the contrary, untreated animals showed a high incidence of bladder tumours with the highest LI and lowest AI and in this group the incidence of aneuploidy increased. The urothelium of rats fed BBN seemed to be in a phase of carcinogenesis sensitive to intravesical chemotherapy and immunotherapy.

Acknowledgements

This study was supported by Grants-in-aid from the FCT, number 12453/2003, Ministry of Science and Technology, Portugal.

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Received March 3, 2006
Accepted April 27, 2006

Artigo #7: DNA study of bladder papillary tumours chemically induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in Fisher rats.

Oliveira PA, Adega F, Palmeira CA, Chaves RM, Colaço AA, Guedes-Pinto H, De la Cruz P LF, Lopes CA.

Int J Exp Pathol. 2007; 88: 39-46.

Int. J. Exp. Path. (2007), 88, 39–46
doi: 10.1111/j.1365-2613.2006.00517.x

ORIGINAL ARTICLE

DNA study of bladder papillary tumours chemically induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in Fisher rats

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INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

Summary

To examine DNA abnormalities in bladder papillary tumours induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in female rats, using image cytometric DNA analysis and cytogenetics. Thirty female rats were exposed to BBN in their drinking water for 20 weeks. One group of 10 animals served as controls. The animals exposed to BBN were killed at a rate of two per week, with the bladder being collected under aseptic conditions and those tumours with exophytic growth removed. The nuclear DNA content of the tumours was evaluated using image cytometric analysis. In two rats part of the tumour pieces was stipulated for culturing. Cytogenetic analysis was performed on at least 30 cells from each cell population and on both tumours. Papillary carcinomas were classified as low grade and high grade. DNA ploidy studies were carried out on 28 low-grade and 21 high-grade papillary carcinomas. Histograms obtained by image analysis showed that a normal urothelium was diploid; 28.6% and 100% of low-and high-grade papillary carcinomas were aneuploid respectively. Both tumours used for cell culture showed multiple numerical and structural chromosome alterations and several marker chromosomes. Image cytometric DNA analysis proved to be a good and reliable method for examining DNA alterations in papillary bladder carcinomas. The present findings establish that the DNA content is statistically different between low-grade and high-grade papillary carcinomas and that deviation from the diploid number is markedly higher in the high-grade ones. In addition, the occurrence of marker chromosomes seems to be related to the aggressiveness of the tumour.

Keywords
chromosome rearrangements, DNA study, image cytometric analysis, papillary carcinomas, rats

Received for publication:
12 June 2006
Accepted for publication:
19 October 2006

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Bladder cancer is currently the most commonly occurring malignant tumour of the urinary tract (Buchumensky *et al.* 1998; Stamouli *et al.* 2004). The prognosis and resulting treatment of bladder cancer are closely related to the histopathological grade and clinical stage of the tumour. About 70–80% of patients with primary bladder cancer exhibit low-grade papillary carcinomas. The remaining 20–30% exhibit solid invasive cancer from the onset (Ariel *et al.* 2004). The incidence rates of bladder cancer are the highest in developed countries (Ioakim-Liossi *et al.* 1999). Major risk factors of this cancer include smoking and contaminating substances generated by the rubber, coal and textile industries, and combustion of fossil fuels (Crawford 1996; Chico & Listowsky 2005).

Nitrosamine-induced tumorigenesis in the rat bladder is a valuable animal model for the study of human bladder cancer. Of the several nitrosamines that can be used to induce tumours in this animal model, N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) is the one most commonly used (Kunze 1979; Becci *et al.* 1981; Fukushima *et al.* 1982). After oral administration of BBN in drinking water, bladder tumours form within 20 weeks. This model is an accurate reflection of those tumours clinically observed in humans; the tumours arise only from the urothelium and they are equivalent to human urothelial carcinoma (Debiec-Rychter *et al.* 1989; Perabo *et al.* 2005).

Current approaches involved in the study of cancer biopathology include cytogenetics, molecular biology, flow cytometry and image cytometric DNA analysis (ICM). ICM has been used to predict the behaviour of several tumours and preneoplastic lesions (Oliveira *et al.* 2005; Cai *et al.* 2006). This technique provides useful information concerning DNA content in a given population of cells (Ioakim-Liossi *et al.* 1999). Cytogenetic analysis plays an important role in clarifying tumour pathogenesis and prognosis (Lee *et al.* 2004).

Numeric chromosome imbalance (aneuploidy) is a hallmark of most solid tumours, whether spontaneous or induced by carcinogens (Pathak & Multani 2006). Data on chromosomal alterations in chemically induced urinary bladder carcinomas are scarce, with only a few studies having been reported (Debiec-Rychter *et al.* 1989; Balakrishnan *et al.* 2002; Vecchione *et al.* 2004). Here, we describe the DNA content in low-grade and high-grade papillary carcinomas and the preliminary cytogenetic analysis in one low-grade papillary carcinoma and in one high-grade papillary carcinoma of the rat urinary bladder.

Materials and methods

Forty-five-week-old female Fisher 344 rats were purchased from Harlan-Interfauna (Amsterdam, the Netherlands). The experiment began after a week of quarantine. The animals were divided into eight groups of five rats, housed in polycarbonate cages at a room temperature of 20 ± 2 °C, with a relative humidity of 50 ± 10%, and with a 12-h light/dark cycle (lights on from 8:00 to 20:00). The rats were maintained with Tecklad diet (Global Diet; Harlan-Interfauna, Barcelona, Spain) and the cages were changed twice weekly to provide hygienic conditions. BBN was purchased from Tokyo–Kasey Kogyo (Tokyo, Japan), and administered in drinking water in opaque bottles at a concentration of 0.05%, over a 20-week period. Rats were fed *ad libitum* throughout the experiment. One group of 10 animals served as control and did not receive any chemical supplements. The experimental design is present in Figure 1. The duration of exposure to BBN was based on data from our previous studies (Oliveira *et al.* 2006). All procedures involving the animals were performed in accordance with the guidelines established after approval by the Portuguese Ethics Committee for Animal Experimentation (Direcção Geral de Veterinária, Approval No. 520/000/000/2003).

After a 20-week exposure to BBN, the animals were killed at a rate of two per week via an intracardiac pentobarbital overdose. Their urinary bladders were removed and opened under aseptic conditions. A portion of each tumour with exophytic growth was fixed in buffered 10% phosphate formalin solution for 12 h. The bladder, liver and kidneys from each rat were removed and fixed using the same solution. After fixation these tissues were routinely processed for paraffin embedding, cut into 2 μm sections, and mounted for haematoxylin and eosin (H&E) staining. Diagnosis, classification and tumour grading were performed using a light microscope, following the World Health Organization's uroepithelial tumour classification criteria (Epstein *et al.* 1998).

Image cytometric DNA analysis methods have been described in detail previously (Oliveira *et al.* 2005) and will

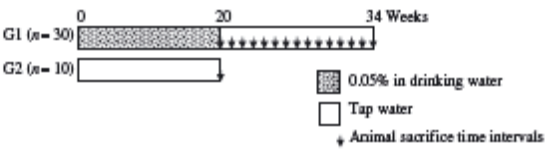

Figure 1 Experimental design.

be summarized here. The nuclear DNA content of cancer cells was measured using the CAS 200 image analysis system (Cell Analysis Systems, Inc., Elmhurst, IL, USA). The image system was first calibrated using the control slide, which contained a known quantity of DNA. Twenty to thirty lymphocytes and a minimum of 100 intact non-overlapping urothelial nuclei were measured and analysed for each case, using the Quantitative DNA Analysis software programme (Cell Analysis System, Inc.). The integrated optical density (OD) of each Feulgen-stained nuclei was considered to be proportional to the amount of DNA present in the nuclei itself. The OD lymphocyte nuclei from each section served as an internal control (diploid reference). The resultant DNA histograms were analysed using previously described methods (Raju *et al.* 1993; Cajulis *et al.* 1995; Reeder *et al.* 1997). The G0/G1 peak was identified visually in each lesion, and the mean, standard deviation (SD) and coefficient of variation (CV) values were calculated. The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of the mean DNA content of the urothelial G0/G1 peak divided by the mean DNA content of the resting diploid lymphocyte G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with values above 5n. Lesions were considered aneuploid only if a separate G0/G1 peak was distinguishable on the histogram and it differed from the reference lymphocyte population by >2SD. A DNA diploid lesion showed a single distinct G0/G1 peak with a DI within 2SD of the control lymphocytes and usually with <1% of 5cER.

The tumour explants were prepared for cell culturing by mincing the solid tissues into small fragments that were transferred to sterile flasks containing the complete medium, in accordance with Chaves *et al.* (2004). The tumour chromosome preparations were made following standard procedures described by Santos *et al.* (2006). Air-dried slides were aged at 65 °C for 5 h, or overnight, and then submitted to standard G-banding procedures using trypsin and fixed in paraformaldehyde, as described earlier (Chaves *et al.* 2004).

A descriptive study was performed for all variables included in this study. Statistical analysis was carried out using the SPSS 12.0 statistical package for Windows. The differences in ploidy frequency within the groups were assessed by using Fisher's exact probability test or the chi-square test. $P < 0.05$ was considered statistically significant.

Results

Clinical observation did not reveal any abnormalities within the groups. No macroscopic or microscopic changes were seen in the liver, lungs and kidneys.

The urothelium was normal in all 10 rats from the control group, 20 weeks after the start of the experiment. A histological classification of bladder tumours is given in Table 1.

Low-grade papillary carcinomas were characterized by variations in the polarity and nuclear size, shape, chromatin texture and cytological atypia. Mitotic activity was low, but was observed throughout the urothelium (Figure 2). High-grade papillary carcinomas were characterized by a completely disordered appearance at low magnification, as well as architectural and cytological abnormalities. Cells

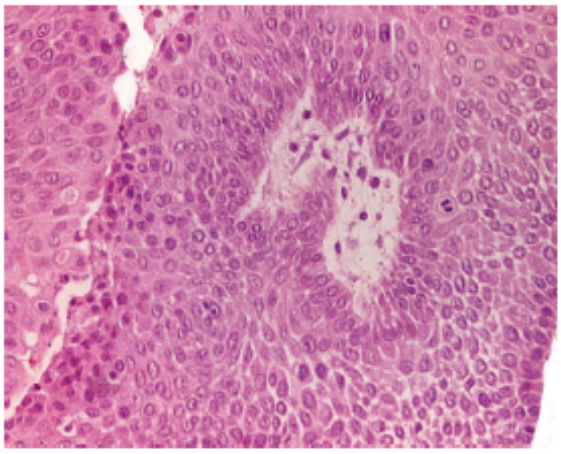

Figure 2 Low-grade papillary carcinoma (H&E, 400×).

Table 1 Frequency of urinary bladder tumours in Fisher 344 rats treated with N-butyl-N-(4-hydroxybutyl) nitrosamine (%)

Group	Treatment	Number of rats	Normal urothelium	Low-grade papillary carcinomas	High-grade papillary carcinomas
1	BBN	30	0	28 (93.3)	24 (80)
2	CTRL	10	10 (100)	0	0

CTRL, control group.

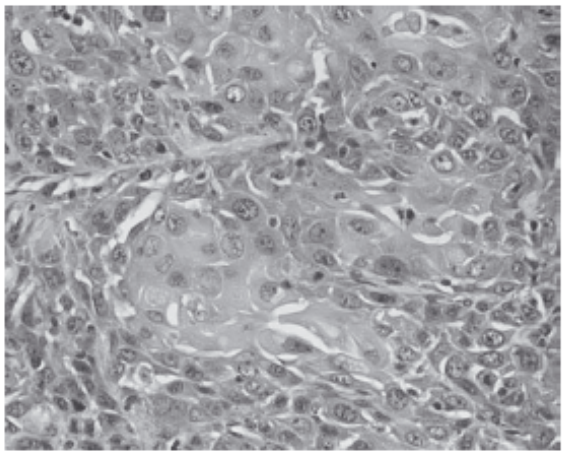

Figure 3 High-grade papillary carcinoma (H&E, 400×).

appear irregularly clustered with the nuclear chromatin clumped together and prominent nucleoli. Mitotic figures were frequently observed (Figure 3).

Image cytometric DNA analysis was successfully performed on 10 normal urothelium, on 28 low-grade and 21 high-grade papillary carcinomas. Figure 4 gives examples of two histograms obtained with CAS 200 from a normal urothelium and from a high-grade papillary carcinoma. Table 2 summarizes the DNA ploidy of the normal urothelium and carcinomas analysed. All normal urothelium exhibited a diploid cellular DNA distribution, 28.6% of low-grade and 100% of high-grade papillary carcinomas were aneuploid. Table 3 summarizes the histological pattern and DNA content of papillary carcinomas at the moment each animal was killed.

One of the tumours analysed using cytogenetics was characterized as a low-grade and the other as a high-grade papillary carcinoma. Most of the cells studied in both carcinomas exhibited very complex karyotypes, displaying a large number of marker chromosomes, identified by their morphological analysis. The complex karyotypes observed together with the aneuploid DNA content analysed using ICM confirmed the malignance of the lesions. In Figure 5, we present an illustrative G-banded metaphase preparation from the high-grade papillary carcinoma. It is possible to observe the marker chromosomes and numerical chromosomal alterations (2n = 84). In this tumour, other cell populations (2n = 79; 2n = 83) were identified (Figure 6). Besides, in the low-grade papillary carcinoma that was cytogenetically analysed, we detected the existence of different cell populations (2n = 41; 2n = 40; 2n = 39), whose diploid chromosome numbers were not far from those in a normal situation (2n = 42).

It should be noted that GTG banding resolution (G-banding produced by trypsin and stained by Giemsa) was poor

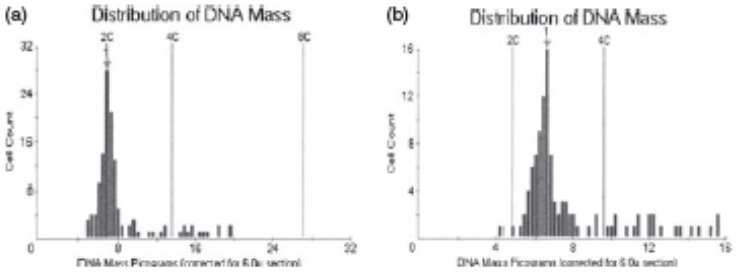

Figure 4 (a) Image analysis DNA histogram of a normal urothelium showing a DNA diploid cell population. (b) Image analysis DNA histogram of a high-grade papillary carcinoma showing a DNA aneuploid cell population.

Table 2 DNA content in normal urothelium and papillary tumours (%)

Group	DNA ploidy	Normal urothelium	Low-grade papillary carcinoma	High-grade papillary carcinoma
1	Diploid	0	20 (71.4)	0
	Aneuploid	0	8 (28.6)	21 (100)*†
2	Diploid	10 (100)	0	0
	Aneuploid	0	0	0

*Statistically different from low-grade papillary carcinoma.
†Statistically different from normal urothelium ($P < 0.005$).

Table 3 Histological pattern and DNA content at the time of killing each animal

	DNA content					
	Histological pattern		Low-grade papillary carcinoma		High-grade papillary carcinoma	
Weeks	Low-grade papillary carcinoma	High-grade papillary carcinoma	Diploid	Aneuploid	Diploid	Aneuploid
20	2	1	1	1	–	1
21	2	2	2	–	–	2
22	2	2	1	1	–	2
23	2	1	2	–	–	1
24	2	1	2	–	–	1
25	2	2	2	–	–	2
26	2	1	1	1	–	1
27	2	1	2	–	–	1
28	2	1	1	1	–	1
29	2	1	1	1	–	1
30	2	1	2	–	–	1
31	2	1	2	–	–	1
32	2	2	1	1	–	2
33	1	2	–	1	–	2
34	1	2	–	1	–	2
Total	28	21	20	8	–	21

Figure 5 Metaphasic preparations obtained from the high-grade papillary carcinoma [observe the marker chromosomes (*), 2n = 84].

with regard to the precise chromosomal identification that is essential for organizing representative karyotypes, as well as for identifying marker chromosomes.

Discussion

The rat has been proposed as an ideal model for studying urinary bladder carcinogenesis, not only because rat papillary carcinomas are similar to those observed in man, but also because spontaneous tumours in the rat urinary bladder are rare, with other spontaneous non-neoplasic changes an uncommon occurrence (Boorman *et al.* 1994).

The biological behaviour of urothelial papillary carcinomas is roughly linked to their histological grade, but there are many cases where precise classification is difficult, requiring the use of complementary methods. In addition, it remains unclear whether there are differences in the type of genetic changes between low- and high-grade papillary carcinomas (Ikemoto *et al.* 2004). To our knowledge, this is one of the first, if not the first, reports describing a DNA study in low- and high-grade papillary carcinomas induced by BBN in rats to include image cytometric analysis and cytogenetics. The correlation between the histological grade and the aneuploid frequency in low-grade papillary carcinomas makes this parameter a valid indicator of the different stages of tumour evolution. Moreover, the statistical difference observed between the frequency of aneuploidy between these two tumours (low-grade *vs.* high-grade) confirmed the possible role of cytometric ploidy as a malignancy indicator. Lesions with morphologically identical cells sometimes exhibit different biological behaviour

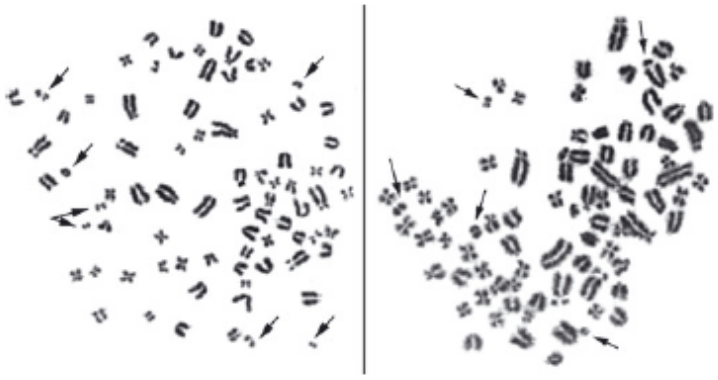

Figure 6 Metaphasic preparations from the high-grade papillary carcinoma [observe the marker chromosomes (arrows), (2n = 79; 2n = 83)].

patterns, so the use of ICM can permit selective examination of each cell and detect the biological aggressiveness of a given tumour (Santos *et al.* 2003). In image cytometry of cancer cells, the DNA content encompasses the chromosome copy number, extra chromosomal fragments and the individual chromosomes, as well as morphometric features of the nuclei (Huang *et al.* 2005). Cytogenetic analysis is, therefore, of great importance in establishing the nature of the alterations that are occurring. The two carcinomas cytogenetically analysed in this study exhibited a large number of marker chromosomes, amongst other chromosome alterations. Numerical chromosome alterations or aneuploidy are one of the most prevalent somatic alterations identified in human tumours (van Velthoven *et al.* 1995; Khaled *et al.* 2004). It has been suggested that aneuploidy instigates tumour progression by enhancing genomic instability, with resulting alterations in cellular phenotypes. Tumours with minimal deviation in chromosome copy number, near diploidy, are clinically less aggressive than those with a greater deviation in chromosome number (Bergers *et al.* 1997; Sen *et al.* 2002). According to Santos *et al.* (2003), as urothelial carcinomas progress, there seems to be a selection of those cells with genetic material gains, conferring them DNA aneuploid status. The low-grade papillary carcinomas (less aggressive) analysed by cytogenetics also displayed cell populations with more regular diploid numbers (2n = 39, 40, 41). The observation of an increasing number of marker chromosomes as the tumours progressed (in the high-grade carcinoma we found cell populations with 2n = 79, 83, 84), leads us to believe that the numerical and structural chromosomal alterations might be important events in the tumorigenesis of bladder papillary carcinomas. This is particularly important as there is gathering evidence that DNA aneuploidy is a key early event in tumorigenesis and may be a cause rather than a consequence of malignancy (Haroske *et al.* 2001; Sudbo *et al.* 2001). Several mechanisms are thought to be responsible for the generation of aneuploid sets of chromosomes: these include failures in cell division, such as defective chromosome separation caused by compromised mitotic checkpoint signalling or centrosome aberrations (Duesberg *et al.* 2005; Schnerch *et al.* 2006). Furthermore, aneuploidy arises to a much higher degree from a tetraploid state when compared with diploid cells (Schnerch *et al.* 2006). Independent of which mechanism causes aneuploidy, it seems that mutagenic and non-mutagenic carcinogens induce genomic instability via aneuploidy (Duesberg *et al.* 2004). In addition to changes in chromosome copy numbers, cancer cells may also experience a change in the size of individual chromosomes because of chromosomal translocations, deletions, and duplications. Cytogenetic studies performed on human urothelial carcinoma have shown several non-random aberrations (Stamouli *et al.* 2004; Fadl-Elmula 2005). The banding methods used in the present study were not sufficient to clarify the exact nature of the chromosome alterations. Further techniques such as *in situ* hybridization with, for example, whole chromosome paint probes, are required to study the tumour evolution by molecularly analysing different cell populations and to track the history of the chromosomal rearrangements which occur during tumour progression.

In conclusion, the cytometric parameters improve existing knowledge on the biological behaviour of low- and high-grade papillary carcinomas of the bladder in rats, and could be used, in association with cytogenetics and histopathological criteria, in the characterization of these tumours. Further studies using molecular cytogenetic techniques in this model will certainly enhance the understanding of subjacent mechanisms associated with rat bladder tumorigenesis.

Acknowledgements

We are grateful to Mrs Ligia Lourenço for her excellent technical assistance. This study was supported by Grants-in-aid from the FCT No. 12453/2003, Ministry of Science and Technology Portugal.

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Artigo #8: Cell proliferation and DNA content in rat urothelial lesions after repeat intravesical instillations of mitomycin C and bacillus Calmette-Guérin.

Oliveira P, Palmeira C, Colaço A, De la Cruz P LF, Lopes C.

Urol Int. 2008; 80: 90-97.

Original Paper

Urologia Internationalis

Urol Int 2008;80:90–97
DOI: 10.1159/000111737

Received: March 14, 2006
Accepted after revision: December 4, 2006

Cell Proliferation and DNA Content in Rat Urothelial Lesions after Repeat Intravesical Instillations of Mitomycin C and Bacillus Calmette-Guérin

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Key Words
Image cytometry analysis · Mitomycin C · Bacillus Calmette-Guérin · Ki-67

Abstract
Aim: To study cell proliferation and DNA content in urothelial lesions identified after repeated intravesical instillations of mitomycin C (MMC) and bacillus Calmette-Guérin (BCG) in normal rat urothelium. ***Material and Methods:*** A total of 45 rats were divided into nine equal groups: those intravesically instilled with MMC; those receiving BCG intravesically, and a control group intravesically instilled with a physiological solution of sodium chloride (SF). Animals were killed 1, 4 or 8 weeks after the last intravesical instillation. An immunohistochemical streptavidin-biotin-peroxidase technique was performed to investigate Ki-67 expression and the DNA ploidy status was measured using static cytometry. ***Results:*** In urothelium exposed to MMC lesions such as simple hyperplasia, dysplasia, carcinoma in situ (CIS), and squamous cell metaplasia were identified. The proliferation index presented values of 11.73, 22.43 and 31.46% in hyperplasias, dysplasias, and CIS, respectively ($p < 0.05$). The frequency of abnormal DNA content amongst those animals exhibiting simple hyperplasias 25% were aneuploid, in the dysplasia 85.2% were aneuploid ($p = 0.041$). CIS were all multiploid, and squamous cell metaplasias were all diploid. Animals treated with BCG and SF presented no urothelial lesions and a diploid DNA content. ***Conclusions:*** The aneuploid and multiploid DNA content and proliferation index observed in urothelial lesions identified after repeated intravesical instillations of MMC reflect a high degree of genomic instability in such lesions which in itself may lead to rapid regeneration of new phenotypes.

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Introduction

Urinary bladder cancer is one of the most common malignancies in the world [1, 2]. More than 70% of bladder cancer patients initially exhibit superficial tumors, although in some cases these eventually progress to invasive disease [3]. Initial therapy for superficial bladder cancer is transurethral resection of all visible tumors [4]. To improve local control and decrease the risk of progression the majority of patients with high-grade or recurrent tumors receive adjuvant intravesical chemotherapy or

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0042–1138/08/0801–0090$24.50/0

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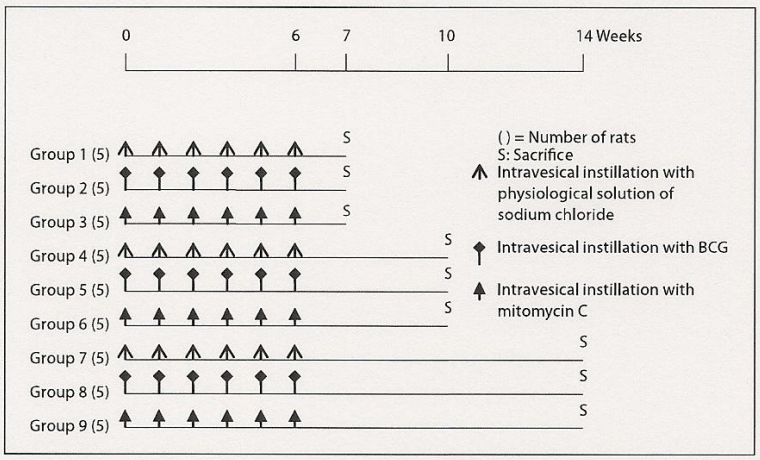

Fig. 1. Experimental design.

immunotherapy [2, 5, 6]. The most commonly used chemotherapeutic and immunotherapy agents are mitomycin C (MMC) and bacillus Calmette-Guérin (BCG), respectively. Studies have recorded an incomplete and variable clinical response to intravesical MMC and associated this compound with mild-to-severe complications related to local bladder toxicity [7]. It is important to study and follow-up the toxic effects of this drug on the normal urothelium to understand the proper evaluation of their efficacy.

Although experimental studies have been performed to histologically evaluate the effects of MMC in rat and mouse urothelium [8, 9], its cell proliferation index and DNA content have not been investigated. To our knowledge, no research has been done to assess the effects of MMC and BCG on the proliferation index and DNA content in urothelial lesions identified after intravesical instillations of these compounds. In this article, we describe and compare the behavior of urothelial lesions identified in the rat urothelium after intravesical instillation of MMC or BCG based on histopathological techniques, immunohistochemistry and DNA cytometry analysis.

Material and Methods

Animals

Five-week-old female Fisher 344 rats (Harlan-Interfauna, Amsterdam, The Netherlands) were selected as the experimental model because of their availability and ease of catheterization. The rats spent 1 week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. They were maintained at 23 ± 2°C, with a 12-hour light/dark cycle and 55 ± 5% humidity. Bedding and cages were changed twice a week. The consumption of food and water was measured at the same time as their body weight was determined. The animals were observed daily to assess their general health. The animals were fed commercial rat food (Harlan Teklad, Glogal Diet) and drank tap water ad libitum. Animal care was provided in accordance with the European Guidelines (86/609/EC) for the care and use of Laboratory Animals. The Portuguese Ethics Committee for Animal Experimentation approved all experiments and procedures carried out on the animals (Direcção Geral de Veterinária, Approval No. 520/000/000/2003).

Experimental Design

A total of 45 female Fisher 344 rats were divided into nine groups, each of 5 rats each. Groups 1, 4 and 7 were intravesically instilled with a physiological solution of sodium chloride (SF) (control groups) once a week for 6 weeks, and sacrificed 1, 4 and 8 weeks after the last instillation. Groups 3, 6 and 9 were instilled once per week for a period of 6 weeks with MMC (Mitomicina-C Kyowa) and sacrificed 1, 4 and 8 weeks later, respectively. MMC was reconstituted with SF according to the manufacturer's recommendation. Groups 2, 5 and 8 were instilled once a week for 6 weeks with BCG and sacrificed 1, 4 and 8 weeks later, respectively (fig. 1). For intravesical immunotherapy a lyophilized Connaught BCG strain containing 81 mg of lyophilized Connaught BCG with at least 1.8×10^8 colony-forming units was used. BCG was also reconstituted with SF, according to the manufacturer's recommendation. Dosing schedules were based on those commonly used in the clinic; 300 μl of MMC solution (1 mg/1 ml) and 300 μl of BCG solution (81 mg/3 ml) [10, 11].

All rats were briefly anaesthetized via an intraperitoneal injection of pentobarbital (45 mg/kg). Pentobarbital has no direct action on the kidney, but may inhibit water diuresis, probably by causing a release of antidiuretic hormone from the pituitary gland [12]. No experimental rats died during the anesthesia or from technical complications. Prior to instillation, micturition of the rats was induced by pressure on the lower abdomen in order to

avoid dilution with urine which would change the concentration of the chemotherapeutic/immunotherapeutic agent. The bladder was catheterized via the urethra with an 18-gauge plastic intravenous cannula. The instillation takes 1 h, after this time the rats' micturition was again induced to eliminate the substance instilled. All the experiments described herein were carried out under aseptic conditions. The rats were turned 90° every 15 min to ensure that the entire bladder was exposed to the substances intravesically instilled. Body temperature was maintained with a homeothermic bandage. After recovery from anesthesia, the rats were placed in standard individual cages for 12 h.

All the rats were sacrificed and their urinary bladders were inflated in situ using an intraluminal injection of buffered 10% phosphate formalin solution for 12 h. The liver and both kidneys were also removed. After the fixation of each bladder, the formalin was removed and the urinary bladder cut sagitally in two. Each half was then cut into four strips for histopathological examination. Sections of 2-μm paraffin-embedded specimens were cut and stained with hematoxylin and eosin (HE) for light-microscopic observation.

Histopathological evaluation was performed under a light microscope. The sections were examined by two researchers. Diagnosis and lesion classification were based on the criteria laid down by the World Health Organization's classification of urothelial tumors [13]. All lesions were re-evaluated using the same guidelines to ensure consistency of diagnosis. Histological changes of the bladder epithelium were divided into the following types: simple hyperplasia, dysplasia, carcinoma in situ (CIS) and squamous cell metaplasia. No histological changes were identified in the liver and kidneys.

Immunohistochemistry

A monoclonal antibody to Ki-67 (M 7248, DakoCytomation) was used. For Ki-67 immunostaining, 2-μm-thick sections were prepared from paraffin blocks. The slides were subsequently deparaffinized in three 5-min changes of xylene and rehydrated through graded alcohol to distilled water. Heat-induced epitope retrieval was performed through pretreatment in a microwave oven (700 W) for 20 min in a 10 mM citrate buffer pH 6.0. After cooling, the sections were immersed in 3% hydrogen peroxide (H_2O_2) and distilled water for 30 min to block endogenous peroxidase activity. Non-specific staining was eliminated by 30 min incubation with normal rabbit serum (X 0902; DakoCytomation). Excess normal serum was removed and replaced by the primary antibody used in 1:20 dilution and incubated overnight (4°C) in a humid chamber. After washing the slides, the sections were incubated with a 1:400 dilution of biotin-labelled anti-mouse secondary antibody (E 0354, DakoCytomation), followed by streptavidin-biotin-peroxidase complex (TS-125-HR, Labvision Corporation) for 10 min. Subsequently, the color was developed with 3,3′-diaminobenzidine tetrahydrochloride with H_2O_2 in PBS buffer for 7 min. Sections were counterstained with Gill's hematoxylin, dehydrated, and mounted. Primary antibodies and biotinylated secondary antibodies were diluted in PBS. Negative controls were carried out by replacing the primary antibody with PBS. Known positive controls were employed.

Immunohistochemical Evaluation

The Ki-67 labeling index (LI) was calculated as the percentage of positive nuclei divided by the total number of cells examined. At least 200 cells per lesion were examined by light microscopy (×400).

Image Cytometry Analysis

Nuclear DNA content of cancer cells was measured using the CAS 200 image analysis system (Cell Analysis Systems, Inc., Elmhurst, Ill., USA). The sections from paraffin-embedded blocks were stained and the resultant DNA histograms were analysed using the aforementioned methods [14]. The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of mean DNA content of the urothelial G0/G1 peak divided by mean DNA content of the resting diploid lymphocytes G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with values above 5c DNA content. Lesions were considered aneuploid only if a separate G0/G1 peak was distinguishable on the histogram and it differed from the reference lymphocyte population by greater than 2 SD (mean ± SD). A DNA diploid lesion showed a single distinct G0/G1 peak with a DI within 2 SD from the control lymphocytes and usually with less than 1% of 5cER. All aneuploid lesions with more than one peak of cells in G0/G1 were classified as multiploid.

Statistical Analysis

Statistical analysis was carried out using the SPSS 12.0 statistical package for Windows. Body weight differences and Ki-67 labeled index per lesion were assessed using ANOVA. For significant values, we used Bonferroni's test. $p < 0.05$ was considered statistically significant.

Results

Table 1 summarizes the results concerning the body weight, water and food intake. The final body weight from groups 6, 7 and 8 showed statistical differences from groups 1, 2 and 3.

Urothelial changes were observed only in rats instilled with MMC. These histological changes in the urothelium consisted of simple hyperplasia, dysplasia, CIS, and squamous metaplasia. Table 2 summarizes the pathologic histology found in each group. When the observation period following MMC instillation increased, the incidence of lesions was similar between groups. Rats in groups instilled with SF and BCG showed no histological changes.

MMC groups showed persistent inflammatory reactions throughout the study. Normal saline groups exhibited minimal focal inflammation of the bladder, and its inflammation decreased over the course of the observation period, all other parameters were normal. BCG groups showed moderate focal inflammation, and the inflammation also decreased over the course of the observation time, all other parameters were normal. No stone formation was apparent in any of the animals.

Normal urothelium consisted of three layers of cells (fig. 2). Simple hyperplasia consisted of focal thickening

Table 1. Data for body weight, food intake and water intake

Group	Treatment	Initial body weight, g[a]	Final body weight, g[a]	Initial food intake g/rat/day	Final food intake g/rat/day	Initial water intake g/rat/day	Final water intake g/rat/day
1	SF 7W	122.53 ± 7.66	175.62 ± 5.88	11.37	11.32	16.96	22.97
2	BCG 7W	120.46 ± 9.71	178.58 ± 11.30	11.93	11.03	16.87	19.97
3	MMC 7W	125.93 ± 8.57	186.54 ± 12.60	11.94	11.97	14.68	17.07
4	SF 10W	129.30 ± 7.01	191.44 ± 7.68	11.74	11.35	18.3	24.73
5	BCG 10W	126.89 ± 9.53	194.28 ± 13.51	10.99	12.01	15.1	22.73
6	MMC 10W	123.04 ± 4.08	200.98 ± 13.93[1]	10.49	11.23	14.4	16.63
7	SF 14W	128.73 ± 6.70	201.74 ± 8.3[1]	11.45	18.1	15.98	21.12
8	BCG 14W	120.65 ± 8.35	203.62 ± 12.7[1, 2]	10.87	19.34	13.9	16.8
9	MMC 14W	121.63 ± 7.77	210.94 ± 13.31[1–3]	12.1	17.98	15.3	19.09

[a] Values are mean values ± SEM.
[1] Significantly different as compared with group 1.
[2] Significantly different as compared with group 2.
[3] Significantly different as compared with group 3.

Table 2. Incidence of urothelial lesions in female Fisher 344 rats instilled with SF, MMC and BCG

Group	Number of animals				
	urothelium	simple hyperplasia	dysplasia	carcinoma in situ	squamous cell metaplasia
1	5 (100)	–	–	–	–
2	5 (100)	–	–	–	–
3	–	2 (40)	3 (60)	4 (80)	1 (20)
4	5 (100)	–	–	–	–
5	5 (100)	–	–	–	–
6	–	5 (100)	5 (100)	4 (80)	2 (40)
7	5 (100)	–	–	–	–
8	5 (100)	–	–	–	–
9	–	4 (100)	5 (100)	5 (100)	2 (40)

Figures in parentheses are percentage.

of the transitional cell lining (fig. 3). Urothelial dysplasia was accompanied by nuclear abnormalities, disturbance in polarity, nuclear crowding and irregularity, increased nuclear size, changes in chromatin pattern and prominent nucleoli (fig. 4). In CIS, we identified a lack of cellular cohesion, larger cells, with a high relation nuclei/cytoplasm and hyperchromatic nuclei (fig. 5). In squamous cell metaplasia, we observed a replacement of transitional epithelium with squamous cells, with a focal or multifocal distribution. Surface cells were keratinized and contained keratohyaline granules, nuclei were round, oval or flattened. We also observed desquamation of the cornified material (fig. 6).

Ki-67 immunostaining was clearly evident as diffuse or dot-like nuclear and nucleolar staining (fig. 7). In groups instilled with SF and BCG no Ki-67 labeled cells were observed. In contrast, in simple hyperplasia, dysplasia, CIS and squamous cell metaplasia, identified in groups instilled with MMC, Ki-67 labeled cells were distributed across every layer of the epithelium. The LI values in each lesion are presented in figure 8. With the transition of the urothelium from simple hyperplasia (11.73 ± 1.87), dysplasia (20.43 ± 3.09) and to CIS (31.36 ± 2.54), the LI values increased, and these differences were statistically different ($p < 0.05$). The mean value of Ki-67 in squamous cell metaplasia was 34.32 ± 3.34.

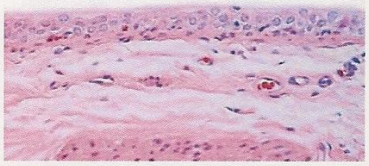

Fig. 2. Normal urothelium. HE. ×40.

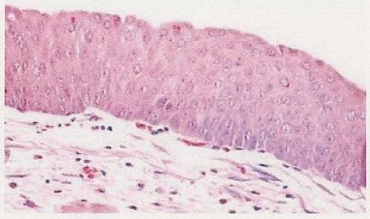

Fig. 3. Hyperplasia. HE. ×60.

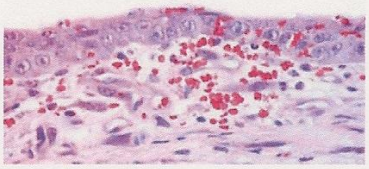

Fig. 4. Dysplasia. HE. ×60.

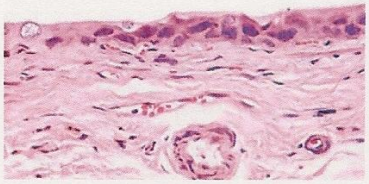

Fig. 5. Carcinoma in situ. HE. ×40.

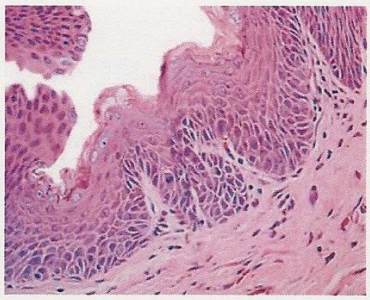

Fig. 6. Squamous cell metaplasia. HE. ×40.

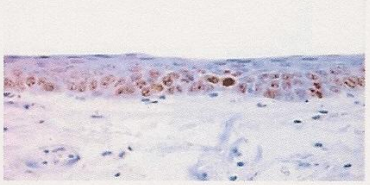

Fig. 7. Ki-67 expression on dysplasia. ×60.

The DNA content on urothelial lesions was evaluated by ICM. This evaluation was successfully performed on 30 normal urothelia (control and BCG groups), 8 simple hyperplasias, 7 dysplasias, 8 carcinomas in situ, and 5 squamous cell metaplasias. The results of DNA content obtained by image cytometry analysis are presented in table 3. Regarding the therapeutic agent used, the DNA content for groups instilled with SF and BCG were diploid. Only MMC-treated animals showed aneuploid and multiploid histograms. Despite the time of observation, the DNA aneuploid pattern frequency increased with the degree of tissue transformation, with the lowest value in hyperplasias and the highest in dysplasias. All squamous cell metaplasia were diploid and all carcinoma in situ were multiploid. This difference was statistically significant ($p = 0.041$) between the two larger groups of lesions, hyperplasias and dysplasias (table 4).

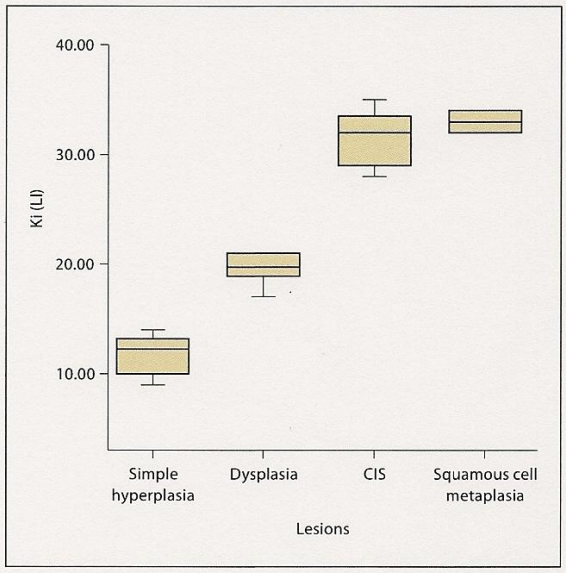

Fig. 8. Mean values of labeling index of Ki-67.

Table 3. Association between DNA ploidy and histological pattern

Group	Histological pattern	Diploid	Aneuploid	Multiploid
1	normal urothelium	5 (100)	0	0
2	normal urothelium	5 (100)	0	0
3	simple hyperplasia	2 (100)	0	0
	dysplasia	1 (33.3)	2 (67.7)	0
	carcinoma in situ	0	0	4 (100)
	squamous cell metaplasia	1 (100)	0	0
4	normal urothelium	5 (100)	0	0
5	normal urothelium	5 (100)	0	0
6	simple hyperplasia	2 (67.7)	1 (33.3)	0
	dysplasia	0	2 (100)	0
	carcinoma in situ	0	0	2 (100)
	squamous cell metaplasia	2 (100)	0	0
7	normal urothelium	5 (100)	0	0
8	normal urothelium	5 (100)	0	0
9	simple hyperplasia	2 (67.7)	1 (33.3)	0
	dysplasia	0	2 (100)	0
	carcinoma in situ	0	0	2 (100)
	squamous cell metaplasia	2 (100)	0	0

Figures in parentheses are percentage.

Table 4. DNA ploidy in hyperplasia and dysplasia

Lesion	Diploid	Aneuploid	p value
Hyperplasia (n = 8)	6 (75)	2 (25)	0.041
Dysplasia (n = 7)	1 (14.8)	6 (85.2)	

Figures in parentheses are percentage.

Discussion

Toxicity is the main dose-limiting factor in cancer therapy. Therefore, it is important to qualitatively assess the side effects induced when comparing different treatments [15]. Several experimental studies have described urothelial lesions observed on rodent bladders after intravesical administration of MMC [8, 9]. However, those lesions were only evaluated by conventional histology; their biological characteristics have not been studied. The results of the present study again provide clear evidence that repeated intravesical instillation of MMC on normal rat urothelium causes histological changes in the urothelium such as the development of simple hyperplasia, dysplasia, CIS, and squamous cell metaplasia. Furthermore, these lesions remained throughout the three periods of observation. No urothelial lesions were recognized in the control group and BCG instilled animals. These observations remained constant at each interval of the study. The morphological changes identified in MMC groups were limited to the epithelial layers, which is consistent with the lack of systemic absorption of MMC [16, 17]. CIS identification after MMC instillation is an important factor. CIS is a serious disease that demands clinical attention. Its course is unpredictable; it may persist or may invade the bladder wall [18]. Long-term results with MMC treatment demonstrated that the percentage of patients suffering a recurrence after 5 or more years is very high [19]. This recurrence may result from urothelial alterations provoked by adjuvant intravesical therapy with MMC, which could evolve into malignant lesions [20, 21].

Ki-67 immunostaining provides an indirect measure of cell proliferation. The monoclonal antibody Ki-67 recognizes the nuclear antigen present in proliferating cells [8, 22, 23]. This nuclear non-histone protein, which is associated with DNA replication and is expressed throughout each phase of the cellular cycle, with the exception of the G0 and early G1 phases, is a good marker of growth in several preneoplastic and neoplastic lesions [24,25]. This antigen is not found during DNA reparation and has a short half-life of 1–2 h. The labeled index of DNA content correlates with the development of several human neoplasias [26, 27]. Cell proliferation is regarded as an important factor in allowing bladder carcinogenesis to alter gene in-

formation and promote tumor development. Although the mitotic index in rat urothelium is normally very low [28, 29], our results showed that Ki-67-positive cells were present at each layer of the proliferative urothelium. As the urothelium changed from simple hyperplasia to dysplasia and to CIS, the LI value increased. Animals intravesically instilled with SF and BCG present no expressivity of the Ki-67 marker; however, MMC increases cell proliferation, and cell proliferation plays a part in fixing mutations, enhancing alterations of gene expression and changing cellular growth control. This difference may be explained by the mechanism of action of these substances. MMC is an antibiotic alkylating agent that inhibits DNA synthesis by the induction of inter- and intrastrand cross-links. Its activity is cell-cycle specific, being most notably high in the late G1 and S phases [30]. The mechanism of action of BCG is still not fully understood, but BCG must be in direct contact with the urothelium. Fibronectin-binding proteins on the surface of the mycobacteria bind to receptors on the surface of the urothelial cells, initiating intracellular signaling which results in cell-mediated anti-tumor activity [31]. MMC toxicity may be related to oxidative stress, MMC enhanced generation of reactive oxygen species that results in the accumulation of oxidative DNA damage or chromosome breakage. This mechanism of action may be responsible for possible late-surfacing effects of MMC therapies [32, 33].

The use of image cytometry analysis requires a lower number of cells allowing the study of small samples and simultaneous analysis of different types of histopathological lesions [34, 35]. Furthermore, microscopic selection of cells enables artefacts to be rejected as well as the detection of aneuploid cells that may represent a small proportion of the whole population [14]. DNA ploidy aberration is related to the cytological and histological features of neoplasia and neoplastic behavior is not always predictable using conventional histologic parameters [36, 37]. Different studies show that cytological and histological neoplastic alterations are preceded by DNA ploidy changes, detected several years before the neoplastic transformation [36, 38]. Aneuploidy reflects a disorder in the genoma correlated with the increased risk of neoplastic transformation [14, 36]. This research shows that cytometric aneuploidy occurs in 25% of cases of simple hyperplasia, 85.2% of dysplasia and in all cases of CIS. The aneuploidy frequency in simple hyperplasia and dysplasia could allow different stages of lesion evolution to be identified. Moreover, the statistical difference observed between the frequencies of aneuploidy of these lesions (simple hyperplasia vs. dysplasia) confirmed the possible role of cytometric ploidy as a marker of malignity. Lesions with morphologically identical cells sometimes exhibit different biological behaviors, so image cytometry analysis can allow selective examination of each cell and detect the biological aggressiveness of a given lesion. These results indicate an increase in the frequency of aneuploidy with an increase in the grade of differentiation. During the development of urothelial lesions induced by MMC there seems to be a selection of those cells with genetic material gains, conferring the DNA aneuploidy. This is particularly important as there is accumulating evidence that DNA aneuploidy is a key early event in tumorigenesis and may be a cause rather than a consequence of malignancy [39, 40].

Degeneration and regeneration of target cells, which are changes induced by both carcinogenic and non-carcinogenic agents are usually observed soon after the administration of several substances. Although these non-specific lesions may be confused with true preneoplastic changes, they stop proliferating when administration is stopped. However, in our experiment the urothelial lesions maintained the same characteristics during the three periods of observation and showed a high proliferation index and aneuploid DNA content. These results demonstrate that cell proliferation study using Ki-67 and image cytometry DNA analysis can provide useful information regarding the biological potential of lesions induced by MMC and suggest that the toxicity and incomplete and variable response to this compound may be caused by high proliferation index and aneuploid DNA content which in itself may lead to rapid regeneration of new phenotypes.

More specifically, it is necessary to understand the molecular impact of repeated MMC instillation in urothelial changes utilizing Western blot and gene array techniques to determine the effect of MMC on gene expression. It is also necessary to conduct further experimental studies to better understand the effects of dose and withdrawal between administrations in lesions induced by repeated intravesical instillation with MMC.

Acknowledgements

This study was supported by Grants-in-Aid from the Fundação para a Ciência e Tecnologia with the number 12453/2003. We would like to express our deepest appreciation to Mrs. Lígia Lourenço for her technical assistance.

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COMPARAÇÃO ENTRE OS MODELOS HUMANO E ANIMAL

Artigo #9: Biological similarities between chemical and natural human bladder carcinogenesis.

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Oncol Letters 2010; 1(2): 373-377.

ONCOLOGY LETTERS 1: 373-377, 2010

Biological similarities between murine chemical-induced and natural human bladder carcinogenesis

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Received September 4, 2009; Accepted November 30, 2009

DOI: 10.3892/ol_00000066

Abstract. The present study investigated the similarities between rodent and human urothelial carcinogenesis models using DNA content, p53 and Ki-67 immunoexpression as surrogate markers of bladder carcinogenesis. Following N-butyl-N-(4-hydroxybutyl)-nitrosamine exposure, 49 human cystectomy specimens of bladder cancer and 53 rat bladder specimens were studied. All of the tumours and adjacent mucosa present in each specimen were evaluated. High similarities were observed between the rodent urothelium carcinogenesis process and the corresponding process in humans, in regards to the histopathological features and biological alteration profile: DNA aneuploidy, p53 overexpression and high proliferative index measured by Ki-67 immunoexpression. Despite these similarities, a higher frequency of alterations was observed in earlier stages in the rat chemical-induced carcinogenesis, namely in 5c aneuploid cells, p53 overexpression and higher Ki-67 labelling index. These results confirm that this experimental animal model is a suitable and reproducible model of bladder carcinogenesis, particularly in regards to high-risk non-invasive and invasive urothelial carcinomas. These features mandate its use in the identification of new molecular targets and evaluation of tumour response to new cytotoxic drugs or drug combinations in bladder cancer therapeutic intervention.

Introduction

The importance of chemical carcinogens in the development of bladder cancer has been well established and generally accepted. This carcinogenesis process occurs as a multistep process. Preneoplastic lesions, differently progressed, but with clonally related genomes, exist prior to *in situ* and invasive carcinoma, and may locally contribute to the independent formation of tumours (1).

Animal models of cancer have been fundamental for the demonstration of this multistep nature (2). Several rodent models have been established to study these different stages of urinary bladder chemical carcinogenesis, namely chemical initiation and promotion, and the stages of progression (3,4). The administration of N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) to mice (5-7) or rats (7-11) is one of the most widely used chemical carcinogens.

The resultant induced tumours in these experimental models have been histologically well studied. However, little is known regarding the biological features and genetic similarities between rodent and human bladder cancer models.

This study aimed to identify biological similarities between human bladder carcinogenesis and rat chemical-induced bladder carcinogenesis by assessing DNA content alterations and p53 and Ki-67 immunoexpression, in order to use this preclinical animal model in future therapeutic experimental studies.

Materials and methods

In this study, we compared biological data collected from our previous studies, in which human (12) and rat (10) bladder samples with premalignant and malignant lesions and adjacent mucosa were studied with regards to histopathology, DNA content, p53 immunoexpression and the Ki-67 labelling index.

Human bladder samples. Consecutive radical cystectomy specimens from 49 patients with previous bladder cancer (non-invasive and invasive urothelial cell carcinomas), consecutively admitted and treated at the Instituto Português de Oncologia do Porto, Portugal, between 1989 and 1996, with available archival material for the image cytometric study and patient consent, were included in the present study. Only high-

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Key words: human, rat, bladder carcinogenesis model, DNA content, p53, Ki-67

risk non-invasive urothelial cell carcinomas received prior intravesical Bacillus Calmette-Guérin treatment. Clinical data were collected from the patient records and the paraffin blocks from the Department of Pathology. All of the lesions present in each cystectomy specimen were studied, including the tumour area and the adjacent mucosa (12).

Rat bladder samples. Fifty-three female Fisher 344 rats were obtained at the age of 5 weeks from Harlan (Amsterdam, The Netherlands). Details concerning the animals, diet, carcinogens and experimental protocol were approved by the Portuguese Ethics Committee for Animal Experimentation (Direcção Geral de Veterinária, approval no. 520/000/000/2003) and are fully described in our previously published study (10). Briefly, animals were randomly separated in five groups. BBN was administered in drinking water at a concentration of 0.05% during a period of 20 weeks to all animal groups, with the exception of group 1, which served as the control and was not given any chemical supplement. After this BBN exposure, animals were maintained on normal tap water until the end of the experiment. Groups 2 and 3 were exposed only to BBN, and animals were sacrificed after an intraperitoneal administration of sodium pentobarbital 1 week (group 2) and 7 weeks (group 3) after BBN exposure. One week after BBN exposure, groups 4 and 5 began intravesical instillations with physiological saline solution (PSS) and mitomycin C (MMC), respectively, once a week for 6 weeks. After this treatment period, the animals were sacrificed. The tumours and adjacent mucosa were studied for each bladder specimen.

For intravesical immunotherapy, the dosing schedules were based on those commonly used in clinical work; 300 μl of MMC solution (1 mg/1 ml) were used (10). The preparation procedures of the bladder for macroscopic examination and tissue processing were previously described (10).

Histopathological analysis. The diagnostic pathology slides were re-evaluated by experienced uropathologists (T.A. for the human series, and C.L. and P.O. for the rat specimens). Pathological staging was performed according to the American Joint Committee on Cancer Staging (13). The histological classification and grading were performed using the criteria from the 2004 World Health Organization (WHO) guidelines (14,15).

DNA content analysis. The DNA content quantification was performed using image cytometric analysis and a CAS 200 Image Analysis System (Cell Analysis Systems, Inc., Elmhurst, IL, USA). All methodological and DNA histogram analysis procedures were previously described in detail (6). Briefly, after the calibration of the image system using a control slide with rat hepatocytes with a known quantity of DNA, 20-30 lymphocytes and a minimum of 100 intact non-overlapping urothelial nuclei were measured and analysed for each case. The G0/G1 peak was visually identified in each DNA histogram, and the mean, standard deviation (SD) and coefficient of variation (CV) values were calculated. The DNA index (DI) describes the relative DNA content of the study population and was defined as the ratio of mean DNA content of the urothelial G0/G1 peak divided by the mean DNA content of the resting diploid lymphocyte G0/G1 peak. The 5cER was also evaluated and defined as the percentage of cells with values >5n. Lesions were considered aneuploid only if a separate G0/G1 peak was distinguishable on the histogram and it differed from the reference lymphocyte population by >2 SD. A DNA diploid lesion showed a single distinct G0/G1 peak with a DI within 2 SD of the control lymphocytes and usually with <1% 5cER.

Immunohistochemical analysis

Human specimens. For the immunohistochemical analysis a standard avidin-biotin peroxidase method was used, as described in a previous study (16). The immunoexpression of p53 and Ki-67 was evaluated with the primary antibodies D07 (1:50 dilution; Dako®) and MIB1 (1:50 dilution; Novocastra®), respectively.

Paraffin-embedded tissues, known to express nuclear p53 (colon carcinoma) and have a high proliferation (lymphoma), were used for titration and positive controls. Negative controls were performed replacing the primary antibody with 2.5% bovine serum albumin in PBS.

Rat specimens. A three-step streptavidin-biotin immunoperoxidase method was used, as described in a previous study (10). The primary antibodies AB-1 (1:50 dilution; Neomarkers-Labvision) for p53 expression and Ki-67 (1:20 dilution; Dako) for proliferative activity, were used. Paraffin sections from colon and breast cancer with known immunoreactivity to p53 and Ki-67 antigens, respectively, were used as positive controls. Negative controls were carried out by replacing the primary antibodies with PBS.

Slides were re-evaluated by two independent observers (T.A. and L.S. for the human cases and C.L. and P.O. for the rat cases) in a blinded fashion, without knowledge of the clinical data. The evaluation method was also described previously (16). The entire lesion was screened to find the region with the maximum fraction of positive- and contiguous-stained nuclei for p53 and the region with the maximum fraction of positivity for Ki-67. The percentage of positive-stained nuclei was scored in this region using a x40 objective. Whenever possible, at least 100 cells were scored in each histological lesion. In the human series, the cut-off values used to distinguish positivity for p53 and Ki-67 were >18 and >32%, respectively, based on studies reported previously (16). In the rat samples, the p53 and Ki-67 immunoexpression was calculated as the percentage of positive nuclei divided by the total number of cells examined (10).

Statistical analysis. The statistical analysis was carried out using the SPSS 15.0 statistical package for Windows (SPSS Inc.). A descriptive analysis was performed for the variables studied. The incidences of differences in molecular alterations between the histological groups were evaluated by the Pearson's Chi-square and the Fisher's exact (when n<5) tests. The mean percentage of positivity of p53 and Ki-67 of the histological types was compared by analysis of variance (ANOVA). $P<0.05$ was defined as statistically significant.

Results

Evaluation of human carcinogenesis. Of the 49 patients included in the present study, 19 cases (38.8%) were classified as high-grade papillary urothelial carcinoma (HGP) and 30

Table I. Frequency of molecular alterations for each histological type observed in the human series.

Variables	Normal n=27 n (%)	HYP n=9 n (%)	DYS n=2 n (%)	CIS n=11 n (%)	HGP n=19 n (%)	INV n=30 n (%)	p-value
DNA ploidy							<0.001
Diploid	26 (96.3)	7 (77.8)	1 (50)	1 (9.1)	7 (36.8)	2 (6.7)	
Aneuploid	1 (3.7)	2 (22.2)	1 (50)	10 (90.9)	12 (63.2)	28 (93.3)	
5cER							<0.001
Without	26 (96.3)	7 (77.8)	1 (50)	1 (9.1)	9 (47.4)	2 (6.7)	
With	1 (3.7)	2 (22.2)	1 (50)	10 (90.9)	10 (52.6)	28 (93.3)	
p53							0.001
Negative	26 (96.3)	8 (88.9)	1 (50)	7 (63.6)	14 (73.7)	15 (50.0)	
Positive	1 (3.7)	1 (11.1)	1 (50)	4 (36.4)	5 (26.3)	15 (50.0)	
Ki-67							<0.001
Negative	26 (96.3)	9 (100)	2 (100)	4 (36.4)	7 (36.8)	8 (26.7)	
Positive	1 (3.7)	0 (0)	0 (0)	7 (63.6)	12 (63.2)	22 (73.3)	

Normal, morphological normal urothelium; HYP, hyperplasia; DYS, dysplasia; CIS, carcinoma *in situ*; HGP, high-grade papillary urothelial carcinoma; INV, invasive urothelial carcinoma.

Table II. Incidence of urothelial histopathological lesions in rats exposed to N-butyl-N-(4-hydroxybutyl)-nitrosamine and treated with Mitomycin C and physiological saline solution.

Group (n)	Normal n=10 n (%)	HYP n=6 n (%)	DYS n=41 n (%)	CIS n=7 n (%)	LGP n=40 n (%)	HGP n=30 n (%)	INV n=10 n (%)
1 (10)	10 (100)	0 (0.0)	0 (0.0)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)
2 (12)	0 (0)	2 (16.6)	10 (87.3)	0 (0)	10 (83.3)	2 (16.6)	2 (16.6)
3 (10)	0 (0)	2 (20.0)	10 (100.0)	0 (0)	10 (100.0)	9 (90.0)	1 (10.0)
4 (11)	0 (0)	1 (9.0)	11 (100.0)	0 (0)	11 (100.0)	11 (100.0)	5 (45.4)
5 (10)	0 (0)	1 (10.0)	10 (100.0)	7 (70)	9 (90.0)	8 (80.0)	2 (20.0)

Normal, morphological normal urothelium; HYP, hyperplasia; DYS, dysplasia; CIS, carcinoma *in situ*; LGP, low-grade papillary urothelial carcinoma; HGP, high-grade papillary urothelial carcinoma; INV, invasive urothelial carcinoma.

(61.2%) as invasive urothelial carcinoma (INV). For all of the tumours the adjacent mucosa was evaluated and showed differential histological patterns dependent on the adjacent tumour. Morphological normal urothelium (n=13, 68.5%), hyperplasia (n=3, 15.8%) and carcinoma *in situ* (CIS) (n=3, 15.7%) lesions were found in the HGP adjacent mucosa, while the INV adjacent mucosa showed morphological normal urothelium (n=14, 46.7%), hyperplasia (n=6, 19.9%), dysplasia (n=2, 6.7%) and CIS (n=8, 26.7%) lesions.

Each one of these histological patterns found in the adjacent mucosa showed the same molecular profile, in regards to the biological variables studied, independently of the adjacent tumour.

Considering each histological type, we compared the distribution of the molecular alterations for the biological variables studied. Statistical significant differences were observed according to the potential of aggressiveness of the lesion (Table I).

Evaluation of rat carcinogenesis. The histopathological lesions observed in each experimental group of rats are described in Table II. No histopathological alterations in urothelial cells were observed in the control group (group 1). The frequency of lesions increased with a longer observation period after BBN exposure. A higher number of HGP and INV cases was observed in the animal group exposed to BBN and submitted to the intravesical instillation of physiological saline solution (group 4). CIS lesions were detected only in animals in group 5, exposed to BBN and treated with MMC.

The molecular alterations found in each histological type are presented in Table III. Despite the period of observa-

Table III. Frequency of molecular alterations for each histological type observed in the rat series.

Variables	Normal n (%)	HYP n (%)	DYS n (%)	CIS n (%)	LGP n (%)	HGP n (%)	INV n (%)	p-value
DNA ploidy								<0.001
Diploid	10 (100.0)	4 (80.0)	9 (56.3)	0 (0.0)	17 (70.8)	0 (0.0)	0 (0.0)	
Aneuploid	0 (0.0)	1 (20.0)	7 (43.8)	7 (100.0)	7 (29.2)	23 (100.0)	3 (100.0)	
5cER								<0.001
Without	8 (80.0)	4 (80.0)	9 (56.3)	0 (0.0)	19 (79.2)	2 (8.7)	0 (0.0)	
With	2 (20.0)	1 (20.0)	7 (43.8)	7 (100.0)	5 (20.8)	21 (91.3)	3 (100.0)	
p53 (positivity)								
mean value	10 (0.0)	6 (20.7)	17 (35.5)	7 (37.4)	34 (36.0)	26 (44.0)	4 (38.9)	<0.001
Ki-67 (positivity)								
mean value	10 (0.0)	6 (16.8)	17 (23.5)	7 (29.3)	34 (23.3)	26 (34.0)	4 (23.9)	<0.001

Normal, morphological normal urothelium; HYP, hyperplasia; DYS, dysplasia; CIS, carcinoma *in situ*; LGP, low-grade papillary urothelial carcinoma; HGP, high-grade papillary urothelial carcinoma; INV, invasive urothelial carcinoma.

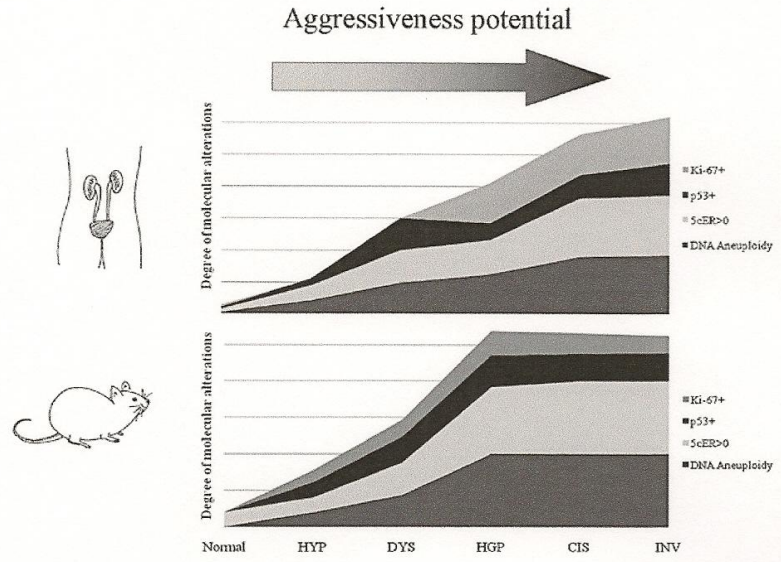

Figure 1. Frequency of molecular alterations for each histological type observed in the human and rodent models. Normal, morphological normal urothelium; HYP, hyperplasia; DYS, dysplasia; HGP, high-grade papillary urothelial carcinoma; CIS, carcinoma *in situ*; INV, invasive urothelial carcinoma.

tion and type of intravesical instillation, the frequency of DNA aneuploidy, 5c aneuploid cells, p53 overexpression and abnormal Ki-67 immunoexpression increased with the degree of aggressiveness of the urothelial lesion.

The comparison between the two cancer models, in regards to the molecular alterations found in each histological pattern, is illustrated in Fig. 1. High similarities were observed between the rodent urothelium carcinogenesis process and the corresponding process in humans, in regards to histopathological features and the profile of biological alterations: DNA aneuploidy, p53 overexpression and high proliferative index measured by Ki-67 immunoexpression. Despite these similarities, a higher frequency of alterations was observed in earlier stages in rat chemical-induced carcinogenesis, namely in 5c aneuploid cells, p53 overexpression and higher Ki-67 labelling index.

Discussion

Cancer involves abnormal cellular growth and appears to have a common molecular basis. Comparing the genetic signatures of human and murine bladder carcinogenesis, William and colleagues (7) found that cell cycle-related genes are involved in the two processes, supporting our options to select DNA content, p53 alterations and Ki-67 labelling index as surrogate markers of bladder carcinogenesis.

The present study found compelling similarities between the rodent urothelium carcinogenesis process and the corresponding process in humans, in regards to histopathological features and the profile of biological alterations: DNA aneuploidy, p53 overexpression and high proliferative index measured by Ki-67 immunoexpression.

The histopathological alterations observed by our group in rat chemical-induced bladder carcinogenesis, namely hyperplasia, dysplasia, low- and high-grade papillary urothelial cell carcinoma, CIS and INV were also observed in natural human bladder carcinogenesis (14,15).

The biological history of bladder cancer shows that DNA aneuploidy is a reflection of genomic instability and chromosomal derangement, and a high aneuploidy level was observed in human advanced bladder cancer (17). *TP53* genetic alterations are associated with tumour stage and grade, and there is a significant association with patient outcome (16,18,19). A high proliferative index is now accepted as a prognostic factor in human bladder cancer improving identification of patients who are at increased risk for disease progression after radical cystectomy (20).

In the experimental orthotopic model described herein, we observed the entire spectrum of lesions that is described in the dual-track pathway of human bladder carcinogenesis: the papillary and non-papillary pathways (21). We also observed that the variation in the frequency of genetic alterations was similar between the rodent (rat) and human cancer models. Thus, the rate of DNA aneuploidy, p53 immunoexpression and Ki-67 labelling index was higher in more aggressive lesions. However, a biological profile similar to that in human invasive tumours was observed in early tumour stages in rats suggesting that this murine model is a beneficial model with which to study the invasive carcinogenesis pathway. William and colleagues also observed that induced murine tumours exhibited more similarities in gene expression to human muscle invasive tumours than non-invasive tumours. Thus, rodent (rat) tumours provide an accurate mechanistic study of genes putatively involved in invasive and metastatic bladder cancer (7).

We also observed a high frequency of biological alterations in high-risk non-muscle invasive tumours, similar to those that occur in human counterparts. This result indicates that this model is also suitable for the study of these particular tumours.

The present and previous results obtained by our group (10,11) emphasize that we now have a technically suitable and highly reproducible model of bladder urothelial carcinogenesis. This model resembles human disease both histologically and in biological behaviour, particularly for high-risk non-muscle invasive and invasive urothelial bladder cancer. Therefore, it is a reliable model with which to identify new molecular targets and evaluate the feasibility and tumour response of new cytotoxic drugs or drug combinations in the field of urologic oncology.

Acknowledgements

This research study was supported by a grant from Associação Portuguesa de Urologia, 2008. The authors are also grateful to Eng. D. Sanders, Portuguese Institute of Oncology, for the English version.

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LISTA SINÓPTICA DOS RESULTADOS RELEVANTES

Pretende-se neste capítulo apresentar, de forma resumida, os resultados mais relevantes observados nos vários artigos que compõem a presente Tese de Doutoramento.

1. Modelo Humano

No conjunto dos 3 artigos incluídos neste tópico verificámos:

a. A citometria de imagem é uma metodologia válida para a determinação do conteúdo de ADN celular, revelando algumas vantagens sobre a citometria de fluxo, nomeadamente: o estudo de amostras pequenas, uma maior sensibilidade para pequenas populações aneuplóides e a possibilidade de permitir analisar separadamente pequenas populações celulares presentes na mesma amostra com base na morfologia. (Artigo #1)
b. Os diferentes parâmetros incluídos na avaliação do conteúdo de ADN são úteis na prática clínica, em especial quando associados a métodos convencionais, como a citologia, ou a outras variáveis biológicas, como o índice proliferativo. (Artigo #1)
c. Durante a progressão das lesões uroteliais, ocorre a acumulação de alterações genéticas que se associam a conteúdos de ADN aneuplóides nas células tumorais, valores mais elevados de células com conteúdo acima de 5c, aumento da imunoexpressão da proteína p53 e aumento da taxa de proliferação avaliada pela imunoexpressão da proteína Ki-67. (Artigos #2 e #3)
d. Existe, no entanto, um subgrupo de CUB invasores com índices proliferativos mais baixos que deverão ser alvo de estratégias terapêuticas diferenciadas. (Artigo #2)
e. O CIS revela um perfil biológico próprio, caracterizado por uma elevada frequência de alterações ao nível do conteúdo de ADN e expressão da p53 e Ki-67, independentemente da sua localização histológica (mucosa adjacente ou à distância do tumor) e tipo de CUB associado. (Artigo #3)

f. A mucosa aparentemente normal, em bexigas com lesões de CIS, tem um perfil de alterações biológicas semelhante ao CIS. (Artigo #3)

2. Modelo Animal

Nos 5 trabalhos desenvolvidos com o modelo animal verificámos:

a. A administração de BBN a uma concentração de 0,05% induz o desenvolvimento de lesões uroteliais, desde a hiperplasia simples aos CUB invasores. (Artigos #4 e #6)
b. Para a indução de CUB, especialmente os papilares de alto grau e invasores, um período de exposição à BBN de 20 semanas é preferível a períodos inferiores. (artigos #4 e #5).
c. Foram detectadas aneuploidias de ADN em lesões hiperplásicas. Este facto realça a maior capacidade da avaliação do conteúdo de ADN em detectar fases iniciais da transformação maligna, relativamente aos critérios estritamente morfológicos. (Artigos #4-#6 e #8)
d. A frequência de lesões uroteliais com conteúdo de ADN aneuplóide associou-se positivamente com o tempo de exposição ao agente carcinogénico BBN. (Artigo #5)
e. As alterações no conteúdo de ADN e na imunoexpressão da p53 e Ki-67 associaram-se de forma positiva com o grau de agressividade das lesões uroteliais. (artigos #4-#8)
f. O tratamento intravesical com BCG e mitomicina C (MMC) revelaram efeitos terapêuticos, verificando-se uma menor frequência de CUB (papilares de baixo e alto grau e invasores) nos animais tratados, comparativamente com o grupo controlo. (Artigo #6)
g. O índice proliferativo avaliado pela Ki-67 foi menor nos animais tratados com MMC e BCG. O facto de se observarem CUB, com menor taxa de proliferação e aneuplóides, em animais tratados, sugere a existência de um grupo resistente à terapia com um agente alquilante ou um imunoterápico. (Artigo #6)

h. Lesões de CIS foram detectadas em animais tratados com instilações repetidas de MMC, com ou sem indução prévia pela BBN. Estas lesões revelaram aneuploidia de ADN, alterações de p53 e aumento do índice proliferativo, sugerindo algum efeito mitogénico e aneuploidogénico deste agente terapêutico. (Artigos #6 e #8)

3. Comparação entre os modelos humano e animal

Neste trabalho de comparação entre os dois modelos de carcinogénese estudados observámos:

a. O modelo experimental em roedores testado revelou todo o espectro de lesões uroteliais descritas nas duas vias de carcinogénese da bexiga no homem: a via dos tumores superficiais papilares e a via dos tumores superficiais planos e tumores invasores. (Artigo #9)
b. As alterações do conteúdo de ADN e imunoexpressão da p53 e Ki-67, observadas no modelo animal, foram sobreponíveis às observadas no modelo humano: as frequências de aneuploidia de ADN, de sobreexpressão da p53 e elevação do índice proliferativo avaliado pelo Ki-67, aumentaram com a agressividade das lesões uroteliais. (Artigo #9)
c. O perfil biológico observado nos CUB invasores humanos, foi detectado em lesões mais precoces no modelo animal de carcinogénese. Este facto sugere um processo de transformação maligna mais rápido e agressivo neste modelo de carcinogénese induzida quimicamente. (Artigo #9)

Discussão e Conclusões

A utilização de modelos animais de carcinogénese urotelial da bexiga, desempenha um papel essencial no desenvolvimento e avaliação pré-clinica de novas estratégias e agentes terapêuticos para o cancro da bexiga [Arentsen, 2009].

O modelo de carcinogénese urotelial da bexiga induzido quimicamente com a BBN em roedores foi já descrito acerca de 3 décadas [Becci, 1981; Bonfanti, 1988; Fukushima, 1982; Oliveira, 2006a]. No entanto, para além do reconhecimento do efeito carcinogénico deste composto genotóxico na bexiga destes animais, pouco se sabia sobre os aspectos histopatológicos e biológicos das lesões uroteliais induzidas quimicamente, e qual o grau de semelhança relativamente às correspondentes lesões no modelo humano.

No conjunto de trabalhos apresentados nesta Tese Doutoral, procurou-se testar e validar este modelo animal de carcinógenese urotelial da bexiga em roedores (murganhos e ratos), comparando as lesões uroteliais observadas com as lesões observadas no modelo humano. Esta comparação baseou-se, quer nos aspectos histopatológicos, quer em características biológicas, nomeadamente, quanto ao conteúdo de ADN, imunoexpressão da proteína p53 e taxa de proliferação avaliada pela imunoexpressão da proteína Ki-67.

Para além de termos observado, no modelo animal testado, o desenvolvimento urotelial de todo o espectro de lesões descritas para o modelo humano, confirmando assim o carácter ortotópico deste modelo experimental, as lesões dos dois modelos foram sobreponíveis, quer histologicamente, quer no perfil de alterações biológicas: as frequências de aneuploidia de ADN, de sobreexpressão da p53 e elevação do índice proliferativo avaliado pela Ki-67, aumentaram com a agressividade das lesões uroteliais. Estas variáveis biológicas revelaram-se instrumentos valiosos para um maior conhecimento das lesões uroteliais e do processo de carcinogénese associado.

O perfil biológico observado nos CUB invasores no homem foi documentado no modelo animal em lesões mais precoces. Este facto, sugere que o processo de carcinogénese química induzido em roedores é responsável pela perturbação da homeostase celular, que se traduz em alterações biológicas uroteliais precoces. Podemos concluir, por conseguinte, que este modelo animal deverá permitir o

estudo de lesões uroteliais sobreponíveis aos carcinomas papilares de alto grau e tumores invasores humanos. William e colegas [William, 2008], ao compararem as assinaturas genéticas associadas aos modelos de carcinogénese no homem e em roedores, verificaram que as alterações encontradas nos tumores dos roedores eram fortemente associadas às alterações dos tumores invasores humanos, o que corrobora as nossas observações.

Verificámos que existem dois grupos particulares de CUB no homem. O primeiro corresponde ao subgrupo de CUB invasores que, ao contrário do perfil biológico dominante, apresentam uma baixa taxa de proliferação. Estes tumores, também documentados no modelo animal, revelaram resistência a um agente alquilante (MMC) e a um agente imunoterápico (BCG), sendo potencialmente resistentes a terapêuticas com recurso a drogas citotóxicas que actuam em células em proliferação. Grossman e colegas [Grossman, 2006] referem, ainda, que este grupo de tumores invasores no homem está associado a pior prognóstico. Estes factos sugerem a necessidade de uma caracterização biológica e genética mais completa, de forma a seleccionar a abordagem terapêutica mais eficaz.

O segundo grupo de CUB corresponde ao carcinoma *in situ* que, para além de ter um elevado risco de progressão, verificámos associar-se a alterações biológicas, nas células uroteliais aparentemente normais próximas e distantes a este tumor, semelhantes ao CIS. Este facto confere um elevado risco de ocorrência de novos tumores. O CIS parece ser um marcador de instabilidade genética urotelial generalizada. A estratégia terapêutica a adoptar nestes casos em que é detectada a presença de CIS, deverá ter em conta este facto de "mucosa urotelial doente".

Os nossos resultados comprovam que o modelo de carcinogénese animal estabelecido é válido e reprodutível, permitindo a sua utilização na pesquisa e identificação de novas estratégias terapêuticas para os CUB superficiais de alto grau e carcinomas invasores.

ESTUDOS FUTUROS

O estudo comparativo, apresentado nesta Tese Doutoral, entre as lesões uroteliais da bexiga induzidas quimicamente em roedores e as correspondentes lesões uroteliais no homem, permite-nos concluir que este modelo animal é válido para estudar o cancro da bexiga e é reprodutível, aspecto fundamental para poder ser aplicado em futuros ensaios pré-clínicos de novas estratégias terapêuticas para este tipo de neoplasia.

Os projectos de investigação nos quais se integra o presente conjunto de trabalhos, têm como propósito clarificar o papel de novos agentes terapêuticos no tratamento dos CUB de mau prognóstico. Este modelo experimental foi utilizado, por exemplo, na avaliação do efeito do sirolimus, droga inibidora da proteína mTOR ("mammalian target of the rapamicin"), nas lesões uroteliais induzidas pela BBN em murganhos.

Em ensaios pré-clínicos pretende-se, com este modelo e com outros estudos, nomeadamente, com linhas celulares de cancro da bexiga humano (xenografos), a detecção de marcadores moleculares associados à acção de drogas nas células tumorais (biomarcadores) e determinar a quimiossensibilidade. Esta investigação pretende validar nos dois modelos (o humano e o experimental) a utilidade de novos agentes com acção anti - angiogénica e linfangiogénica, entre outras.

Pretendemos confirmar, no modelo animal, o significado biológico da lesão de CIS com base nos dados observados no homem, ou seja, um marcador de instabilidade genética generalizada no urotélio. Caso se confirme esta semelhança, teremos, também, disponível, um modelo experimental para avaliação de esquemas terapêuticos mais adequados ao tratamento desta lesão superficial de alto grau.

O subgrupo de CUB invasores com índices proliferativos mais baixos, observados nos dois modelos de carcinogénese, é outro grupo de tumores que necessita de terapêutica diferenciada. Uma melhor identificação das alterações genéticas associadas a este grupo de CUB, poderá permitir a utilização de terapêuticas dirigidas a alvos moleculares úteis.

A aplicação do modelo animal aqui testado, a par do desenvolvimento de animais transgénicos e linhas celulares manipuladas geneticamente, irá contribuir,

decerto, para um maior conhecimento do processo de carcinogénese urotelial e para identificação de alvos terapêuticos e drogas associadas, permitindo uma terapêutica personalizada.

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