Ana Carolina Cuzzuol Fracalossi

CARCINOGÊNESE BUCAL QUIMICAMENTE INDUZIDA PELA 4-NITROQUINOLINA 1-ÓXIDO EM RATOS:

possíveis biomarcadores envolvidos em sua patogênese

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do título de Doutor em Ciências

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ESCOLA PAULISTA DE MEDICINA

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DEDICATÓRIA

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Assim seja!

Amém!

“Algumas vezes você se pergunta...

Tudo isso vale a pena?

Devo continuar com tudo isso?

E por quanto tempo?

Você vive situações inesperadas, mas isto faz parte da sua vida.

Ou você encara, ou desiste e para!

Eu gosto demais do que eu faço...

Eu não posso parar!”

“Seja quem você for, tenha sempre como meta muita força, muita determinação e sempre faça tudo com muito amor e com muita fé, que um dia você chega lá, de alguma forma você chega lá!”

Ayrton Senna

Fui à floresta porque queria viver profundamente

E sugar a essência da vida.

Eliminar tudo o que não era vida.

E não, ao morrer, descobrir que não vivi.

(**Henry David Thoureau**)

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Sumário

Lista de Figuras

.Lista de Tabelas

Lista de Abreviatura

% - Porcentagem

°C – Graus Celsius

4NQO – 4-nitroquinoline 1-oxide

Alpha-SMA – Alfa actina de músculo liso

DMBA - Dimetilbenzantraceno

DNA – Ácido desoxirribonucléico

g – Gramas

h – Horas

H-ras - Harvey rat sarcoma viral oncogene

K-ras - Kirsten rat sarcoma viral oncogene

mg - Miligramas

mL/kg – Mililitros por kilo de peso

PCNA – Antígeno nuclear de proliferação celular

PCR – Reação de polimerização em cadeia

ppm – Partes por milhão

Rpm – Rotações por minuto

TBE - Tris/Borato/EDTA

TNM – Classificação dos tumores malignos – tumor/ linfonodo/metástase

UV – Ultravioleta

V/min – Volts por minuto

VEGF – Fator de crescimento endotelial vascular

Resumo

Objetivos: este trabalho teve como objetivo investigar mutações nos éxons 1 e 2 do *H-ras* e *K-ras*, bem como, a imunoexpressão do H-ras, Ki-67, alfa-SMA, metaloproteinases 2 e 9 (MMP-2 e MMP-9) e a via Wnt/β-catenina (Wnt1, Frizzled-1, Wnt5a, Frizzled-5 e β-catenina) durante a carcinogênese bucal quimicamente induzida pela 4-nitroquinolina 1-óxido (4NQO) em ratos. **Material e Métodos**: Ratos Wistar machos foram distribuídos em três grupos de 10 animais cada e tratados com 50 ppm de 4NQO, via bebedouro, durante 4, 12 e 20 semanas. Dez animais foram utilizados como controle. **Resultados**: apesar da ausência de alterações histopatológicas no epitélio após 4 semanas de exposição ao cancerígeno, Ki-67 e Wnt1 mostraram alta expressão no epitélio oral "normal"; expressão de Ki-67, MMP-2 e MMP-9 foi detectada tanto nas lesões pré-neoplásicas quanto nos carcinomas espinocelulares bem diferenciados induzidos após 12 e 20 semanas de tratamento com a 4NQO, respectivamente; alfa-SMA revelou-se diferencialmente expressa nos carcinomas espinocelulares. Nenhuma diferença significativa ($p>0.05$) foi encontrada na expressão de H-ras, Frizzled-1, Frizzled-5 e β-catenina, em todos os momentos avaliados. Do mesmo modo, nenhuma mutação foi encontrada nos genes *H-ras* e *K-ras*. **Conclusões:** de acordo com os resultados encontrados, o aumento do status proliferativo a partir do Ki-67 e expressão de Wnt1, MMP-2 e MMP-9 estão associados ao risco e à progressão do câncer bucal, enquanto que mutações no gene *Ras* parecem não estar envolvidas na carcinogênese lingual induzida pela 4NQO.

INTRODUÇÃO

1. INTRODUÇÃO E JUSTIFICATIVA

1.1 Considerações Gerais

O câncer bucal compreende lesões malignas da região de lábio e cavidade oral propriamente dita (mucosa bucal, gengivas, palato duro, língua e assoalho da boca). Representa a sexta neoplasia mais prevalente no *ranking* mundial, a terceira em países em desenvolvimento (Massano et al. 2006). A doença apresenta incidência elevada, particularmente, em países como Índia, Vietnã e Brasil, constituindo aproximadamente 25% de todos os tipos de cânceres (Magrath & Litvak 1993; Sharma et al. 2010). Entretanto, a sua incidência também tem aumentado nos Estados Unidos e na Europa nas últimas décadas (Rodrigues et al. 1998).

Os principais fatores exógenos envolvidos na tumorigênese bucal são álcool e tabaco (Hahn et al. 2002). Isoladamente, o consumo destas drogas aumenta em duas a três vezes o risco para o desenvolvimento do câncer e quando associados, o risco aumenta em, aproximadamente, 15 vezes (Mashberg et al. 1993; Castellsagué et al. 2004).

Morfologicamente, o carcinoma espinocelular compreende 90% dos casos de cânceres de boca (Massano et al., 2006; Baykul et al., 2010), sendo os locais mais acometidos a língua, o lábio inferior e o assoalho bucal (Silverman 2001; Kademani et al. 2005). A evolução desta neoplasia é precedida por fases pré-malignas, microscopicamente caracterizadas pelas displasias que variam de leve à grave. Apesar das displasias indicarem maior propensão para a malignidade, nem toda lesão displásica evolui para lesão maligna. Isso porque, o real mecanismo envolvido na transformação

maligna continua desconhecido. Mediante a isto, explorar os mecanismos patobiológicos envolvidos na carcinogênese bucal e identificar possíveis biomarcadores da tumorigênese em boca é de fundamental importância. Neste contexto, a aplicação de modelos experimentais para o estudo da carcinogênese tem sido uma alternativa promissora de alguns grupos de pesquisa (Fassoni et al. 1993; Li et al. 1999; Nishimura, 1999; Okazaki et al. 2002; Chen et al. 2003; Schwartz et al. 2004; Ribeiro et al. 2004 a, b; Vered et al. 2005).

1.2 Carcinogênese Bucal Experimental

Os modelos de carcinogênese bucal tiveram início com as investigações de Salley (1954) que demonstrou que os carcinomas espinocelulares poderiam ser produzidos na mucosa jugal de hamsters, por meio de múltiplas aplicações locais de agentes cancerígenos químicos. Os tumores desenvolvidos variavam de papilomas a carcinomas espinocelulares com metástase para linfonodos. Posteriormente a este estudo, Salley (1957) mostrou a evolução da carcinogênese e relatou os quatro estágios precedentes à neoplasia induzida: inflamação, degeneração, regeneração e hiperplasia. Em 1961, com o objetivo de contribuir para a padronização de métodos para a avaliação da carcinogênese bucal, Morris observou que animais com três, seis e nove meses de idade não apresentavam diferença no período de latência para desenvolvimento de tumores.

Atualmente, os modelos experimentais comumente empregados no estudo da carcinogênese bucal quimicamente induzida são: em hamsters com DMBA (dimetilbenzantraceno), hidrocarboneto aromático policíclico com potencial cancerígeno bem estabelecido (Fassoni et al. 1993; Li et al. 1999; Chen et al. 2003), como o

benzo[a]pireno, um dos componentes do cigarro (Schwartz et al. 2004); em ratos, empregando a 4-nitroquinolina 1-óxido (4NQO), agente alquilante utilizado em modelos de carcinogênese experimental na mucosa bucal (Nishimura, 1999; Okazaki et al. 2002; Ribeiro et al. 2004 a, b; Vered et al. 2005), que é o sistema-teste adotado pela maioria dos pesquisadores por apresentar lesões pré-neoplásicas e neoplásicas semelhantes àquelas da cavidade bucal de seres humanos (Nauta et al. 1996; Dayan et al. 1997). Este modelo está baseado no processo de múltiplas etapas, caracterizado pela iniciação, promoção e progressão (Ribeiro et al. 2004 a, b; 2005; 2007a, b; 2008; 2009; Silva et al. 2007; Minicucci et al. 2009; 2010; Fracalossi et al. 2010; Fracalossi & Miranda 2010).

1.2.1 4-Nitroquinolina 1-óxido (4NQO)

A 4-Nitroquinolina 1-óxido (4NQO) é um derivado da quinolina, cujo efeito cancerígeno foi primeiramente observado por Nakahara et al. (1957). Subsequentemente, a 4NQO foi utilizada em boca de hamster para indução de carcinomas (Fujino et al. 1965).

Os mecanismos pelos quais esta substância exerce sua atividade cancerígena na mucosa bucal têm sido muito investigados. A 4NQO exerce estresse oxidativo intracelular, sendo que os subprodutos de sua metabolização intracelular ligam-se à molécula de DNA, predominantemente nos resíduos guanina. Este efeito parece semelhante aos dos cancerígenos presentes no tabaco (Kanojia & Vaidya 2006).

Na indução do estresse oxidativo intracelular (Nunoshiba et al. 1993), a 4NQO leva à formação de espécies reativas de oxigênio (ERO) tais como radical superóxido ou peróxido de hidrogênio (Ishizawa & Endo, 1967; Sugimura et al. 1968; Hozumi, 1969). O ciclo de redução é um processo em que há redução enzimática de um elétron do

composto seguido pela auto-oxidação em presença do oxigênio molecular. O oxigênio é reduzido ao íon superóxido que pode levar à formação de outras espécies reativas derivadas do oxigênio, tais como peróxido de hidrogênio (H_2O_2) e radicais hidroxil. Radicais de ânion anidro dos compostos nitroaromáticos e nitroheterocíclicos são mediados na redução enzimática destes compostos para aminas e aminas hidroxil (Fig. 1) (Nunoshiba et al. 1993).

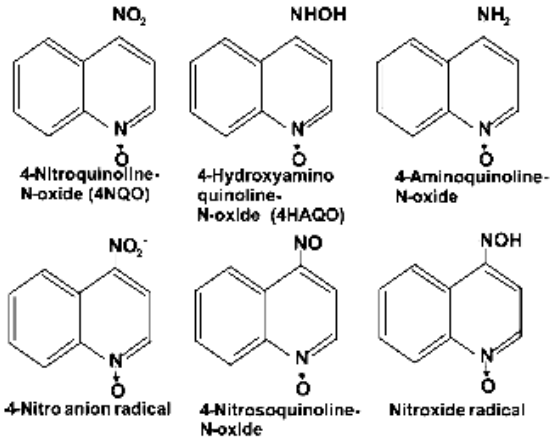

Fig. 1: Estrutura da 4NQO e seus metabólitos (Koontongkaew et al. 2000).

A ação cancerígena da 4NQO é iniciada pela redução enzimática de seus grupos amina. Assim, a mesma é reduzida a 4-hidroxiaminoquinolina 1-óxido (4HAQO) pela nitroredutase NADH e pela redutase quinona NAD(P)H (Benson, 1993). A 4HAQO é um metabólito da 4NQO implicado na formação de adutos. Pode ser ainda metabolizada e acetilada pela sintetase seril-tRNA a um complexo enzimático seril-AMP (Friedberg et al. 1995). Este complexo pode também produzir grupos quinolina na molécula de DNA.

Resistência à genotoxicidade e citotoxicidade induzidas pela 4NQO é influenciada pela proteína multidroga resistente (MPR) e pela glutationa S-transferase P (GSTP1-1). A 4NQO é um substrato para a glutationa S-transferase (GSTs) incluindo GSTP1-1, que

utiliza glutationa para conjugação. O conjugado resultante é chamado glutationa S - 1-óxido quinolina (QO-SG). Este pode ser liberado da célula pela MPR (Friedberg et al.1995).

Empregando a 4NQO radioativa para análise dos adutos 4NQO-DNA e ensaios de citotoxicidade, demonstrou-se que MPR e GSTP1-1 protegem contra formação de adutos e citotoxicidade (Morrow et al. 1998). Entretanto, a atuação individual destas proteínas promove limitada ação protetora à 4NQO (Morrow et al. 1998). Estes resultados demonstram que GSTP1-1 e MPR podem ter importante papel ao limitar os eventos de iniciação e progressão da carcinogênese impostos pelo agente químico.

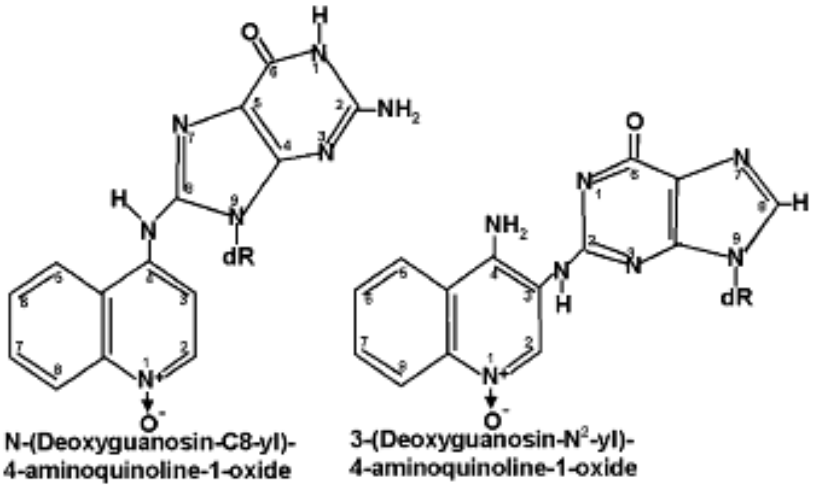

Fig 2. Estrutura dos adutos de guanina no DNA (Friedberg et al. 1995).

1.3 Oncogenes

O câncer representa um distúrbio genético que pode ser provocado por mutação ou deleção em alguns genes que codificam proteínas capazes de estimular e controlar o crescimento e a divisão celular; estes genes mutados são denominados oncogenes. Nesse grupo, encontram-se os membros da família *Ras*.

Os oncogenes da família *Ras* foram pioneiramente identificados em retrovírus murinos associados a sarcomas de Harvey (H) e Kerstein (K). Um terceiro gene foi detectado em neuroblastomas humanos (*N-Ras*). Tais genes codificam proteínas homólogas de peso molecular de 21kDa, denominadas de p21Ras, as quais são reguladoras da função intracelular relacionada à transdução de sinal (Waldmann & Rabes, 1996). A p21Ras está ligada à superfície interna da membrana plasmática e tem homologia com membros da família das proteínas G. Biologicamente, possui atividade intrínseca via GTPase (família de enzimas hidrolases que se ligam e hidrolizam o GTP), uma vez que se torna inativa quando o gene *Ras* é mutado, promovendo contínua propagação de sinais proliferantes desencadeando contínua divisão celular (Waldmann & Rabes, 1996).

Os genes da família *Ras* encontram-se mutados nos cânceres de pâncreas, colorretal, endométrio, biliar, pulmão, bexiga (Minamoto et al. 2000; Tsujioka et al. 2010) e também têm sido investigados no câncer bucal (Hoellering et al. 1989; Saranath et al. 1991; Warnakulasuriya et al. 1992; Suzui et al. 1995; Das et al. 2000; Caulin et al. 2004; Sathyan et al. 2007). No que diz respeito à carcinogênese oral experimental induzida pela 4NQO, poucos autores avaliaram mutações na família *Ras* em lesões displásicas e em carcinomas espinocelulares (Tsuda et al. 2007; Kim et al. 2002; Suzui et al. 1995). Neste

contexto, o momento exato em que as mutações nos genes *Ras* ocorrem na carcinogênese bucal induzida pela 4NQO não foi divulgado até o momento.

1.4 Progressão Tumoral

A proliferação celular é considerada um dos mais importantes mecanismos biológicos na oncogênese, haja visto que a atividade proliferativa contribui tanto nas fases de promoção como de progressão (van Diest et al. 1998). A proteína Ki-67 representa um marcador de proliferação celular, presente durante o ciclo celular, com exceção da fase inicial G1 (Tomasek et al. 2002). Por essa razão, o anticorpo anti-Ki-67 tem sido utilizado em estudos com o propósito de investigar a atividade proliferativa em diferentes órgãos como o pulmão, cérebro, próstata e mucosa bucal (Oshima et al. 2005; Smilek et al. 2006; Drakos et al. 2007; Kim et al. 2007; Kudrimoti et al. 2007; Steinau et al. 2007). O índice elevado deste marcador em carcinomas espinocelulares bucais tem sido correlacionado com progressão e mau prognóstico da doença (Matsumoto et al. 1999; Schoelch et al. 1999).

Por se tratar de uma doença complexa e de múltiplas vias, outros parâmetros devem ser considerados em detrimento da genômica funcional na investigação de tumores malignos. Miofibroblastos, originalmente identificados nos tecidos de granulação em atividade contráctil durante o processo de reparo tecidual, têm sido eleitos um dos protagonistas na reação desmoplásica estromal em diferentes tumores epiteliais (Desmouliere et al. 2004). Adicionalmente, estas células têm sido correlacionadas ao processo de invasividade tumoral, em decorrência da sua habilidade em produzir tecido conjuntivo, bem como proteínas da matriz extracelular (Lewis et al. 2004; Barth et al. 2004;

Vered et al. 2005). De fato, estudos têm levantado à hipótese que os miofibroblastos são importantes sinalizadores entre epitélio e estroma, conforme demonstrado em publicações envolvendo alguns cânceres humanos, tais como os de mama, cérvix e colorretal tanto *in vitro* como *in vivo* (Sappino et al. 1988; Cintorino et al. 1991; Martin et al. 1996). Todavia, a real significância desse mecanismo durante a carcinogênese bucal experimental não fora reportada até o presente momento.

Ainda em relação aos mecanismos de progressão tumoral, destacam-se também algumas metaloproteinases, um amplo grupo de enzimas dependentes de zinco, responsáveis pela degradação de componentes da matriz extracelular, incluindo, colágeno, gelatina, fibronectina, laminina e proteoglicana em processos patológicos e fisiológicos tais como embriogênese, angiogênese, remodelação tecidual, invasão tumoral e metástase (Coussens et al. 2002). A MMP-2 também chamada colagenase tipo IV, cliva colágenos dos tipos IV, V, VII, X e gelatina tipo I, enquanto, MMP-9, também denominada colagenase IV de 92-kDa ou gelatinase B, degrada colágeno ósseo. Ambas estão associadas à invasão, ao crescimento do tumor e à metástase em carcinomas sendo, portanto, importantes para determinar o comportamento da célula neoplásica (Nelson et al. 2000; Xu et al. 2005; Deryugina & Quigley 2010).

1.5 Família Wnt

A família de proteínas Wnt representa um grupo de glicoproteínas, que têm papel na regulação, proliferação e diferenciação de células epiteliais envolvidas na tumorigênese de vários tecidos (Akiyama, 2000; Seidensticker & Behrens, 2000; Yamaguchi, 2001). Os genes Wnt têm sido classificados em grupos funcionais que regulam separadamente vias

de sinalização intracelular. São descritas duas principais vias: canônica e não-canônica. A primeira inclui um complexo multiproteico que engloba polipose adenomatosa familiar (APC), glicogêncio sintase quinase 3 (GSK3) e axina que fosforilam os resíduos serina e treonina no N terminal da ß-catenina, na ausência de ligantes Wnt. Na presença de ligantes do Wnt, através de receptor Frizzled (FZD) e receptores lipídios de baixa densidade (LRP5/6), o complexo inativa GSK3 e causa sua dissociação da Axina, prevenindo a degradação da ß-catenina (He et al. 2004). Assim, a ß-catenina se acumula no citoplasma e é translocada para o núcleo e se liga ao fator de células T (TCF) e a fatores de transcrição da família do LEF (lymphoid enhancer-biding protein), ativando a transcrição de genes alvo como c-myc, ciclina D, VEGF, entre outros, relacionados ao crescimento, migração, diferenciação, angiogênese e apoptose (Logan & Nusse, 2004). Na segunda via, a sinalização é realizada por meio de Frizzled, independente de LPR5/6. Esta via promove mudanças no citoesqueleto e na ativação de pequenas GTPases Rho e Rac (Habbas et al. 2005).

Dentre as glicoproteínas da via Wnt, a Wnt1 parece ter importante papel nas áreas de infiltração e diferenciação, mostrando imuno-expressão aumentada no fronte de invasão dos carcinomas da região da cabeça e pescoço (Lo Muzio, 2002). A via de sinalização Wnt1 é mediada via interação entre a ß-catenina e membros da família LEF/TCF dos fatores de transcrição.

A Wnt5a parece ser específica para a via não-canônica e tem sido associada a inúmeros tumores (Smalley & Dale, 1999; Yu et al. 2007). Wnt5a tem sido identificada como um supressor de tumor em carcinomas de tireóide, câncer de mama e tumores hematológicos (Kremenevskaja et al. 2005; Liang et al. 2003; Jonsson et al. 2002). Em

contraste, a expressão de Wnt5a tem estimulado a migração e invasão celular em carcinomas de mama, câncer gástrico e melanoma (Lewis et al. 2005; Pukrop et al. 2006; Kurayoshi et al. 2006).

No que diz respeito à carcinogênese bucal induzida pela 4NQO, expressão das proteínas Wnt1 e Wnt5a não havia sido investigada até o momento.

Em suma, considerando as múltiplas vias e a complexidade da carcinogênese, buscou-se explorar as proteínas e os genes anteriormente descritos em três artigos independentes, que serão apresentados na íntegra no decorrer desta tese.

OBJETIVOS

2. OBJETIVOS

Considerando as múltiplas vias que podem estar alteradas na carcinogênese e o modelo experimental de indução do câncer por meio da 4NQO, a proposta deste trabalho foi analisar as múltiplas etapas deste processo na língua de ratos, tendo como parâmetros:

1. Caracterizar as alterações morfológicas macro (-) e microscópicas;
2. Identificar as possíveis mutações nos éxons 1 e 2 dos oncogenes *H-ras e K-ras*, bem como a expressão do H-Ras, ki-67 e alfa-SMA;
3. Analisar a imuno-expressão dos genes relacionados às metaloproteínases - MMP2 e MMP9;
4. Investigar a expressão das proteínas da via Wnt/ß-catenina - Wnt1, Frizzled-1, (FZD1), Wnt5a, Frizzled-5 (FZD5) e β-catenina.

ARTIGOS

3. ARTIGOS

3.1. ARTIGO 1 ACEITO PARA PUBLICAÇÃO NO JOURNAL OF ORAL PATHOLOGY AND MEDICINE – Fator de impacto: 2.144

Ras gene mutation is not related to tumor invasion during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide

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Short title: Ras Mutation in rat tongue cancer

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Abstract

BACKGROUND: The aim of this study was to investigate if mutations in the genes *H-ras* and *K-ras* were related to mechanism of invasion as a result of the immuno-expression of H-Ras, Ki-67, alpha-SMA and VEGF during 4-nitroquinoline 1-oxide (4NQO)-induced rat tongue carcinogenesis.

METHODS: Male Wistar rats were distributed into three groups of 10 animals each and treated with 50 ppm 4NQO solution through their drinking water for 4, 12, and 20 weeks. Ten animals were used as negative control.

RESULTS: Although no histopathological abnormalities were induced in the epithelium after 4 weeks of carcinogen exposure, Ki-67 was overexpresssed in the 'normal' oral epithelium. In pre-neoplastic lesions at 12 weeks following carcinogen exposure, the levels of Ki-67 were increased ($p<0.05$) when compared to negative control. Ki-67, alpha-SMA and VEGF were also overexpressed in squamous cell carcinomas induced after 20 weeks of treatment with 4NQO. No significant statistically differences ($p>0.05$) were found to H-Ras protein expression for all experimental periods evaluated that corresponded to normal oral mucosa, hyperplasia, dysplasia and squamous cell carcinomas. In the same way, no mutations in *H-ras* or *K-ras* genes were found.

CONCLUSION: Our results support the idea that expression of Ki-67 plays a crucial role during malignant transformation being closely related to neoplastic conversion of the oral mucosa cells. However, it seems that mutations in the *Ras* genes are not involved to experimental tongue carcinogenesis induced by 4NQO.

Key words: oral squamous cell carcinoma; 4-nitroquinoline 1-oxide, immunohistochemistry, gene *Ras* mutation.

Introduction

Oral cancer (oral squamous cell carcinoma, OSCC) is the sixth most common human malignancy, with a 5-year mortality rate of approximately 50% (1,2). The therapeutic modality currently offered to OSCC patients is based on traditional stage-predicting indices (based mostly on the TNM criteria) and on histological grading. Unfortunately, these predictors are subjective and relatively unreliable, since there are tumours with identical staging and grading with distinct behavior. Thus, there has been an evergrowing effort dedicated to the basic research focusing on the identification of biological indicators for the diagnosis of its biological nature and aggressiveness.

The most used animal models in oral cancer research are the hamster buccal pouch by fat-soluble 7,12 dimethylbenzanthracene (DMBA), and the rat tongue by water-soluble 4-nitroquinoline 1-oxide (4NQO). Considering that one of the most important routes of oral carcinogens is through liquid containing water-soluble carcinogens, 4NQO is well suited in examining the role of proteins in experimental oral carcinogenesis (3-5). Based on the multistep process of carcinogenesis characterized by initiation, promotion and tumor progression, chronic administration of 4NQO in drinking water simulates rat tongue carcinogenesis in a similar way to that which develops in humans (6-11).

Alterations affecting the expression or function of genes controlling cell growth and differentiation are considered to be the main cause of cancer. Molecular cancer research aims to identify the genes that are altered in the various tumor types as well as to elucidate their role in carcinogenesis. A family of genes that is frequently found to harbor a mutation in human tumors is that of the *Ras* family (12). The family consists of three functional genes, *H-ras*, *K-ras*, and *N-ras*, which encode highly similar proteins with molecular

weights of 21,000 (p21ras) (12). The functional and structural resemblance of the ras proteins with the G-proteins controlling adenylate cyclase has led to the proposal that normal p21ras proteins are involved in the transduction of external stimuli, most likely induced by growth factors or factors involved in cell differentiation. Mutated ras proteins, however, have lost the ability to become inactivated and thus stimulate growth or differentiation autonomously (13). The real prominence of these genes in pathogenesis of oral cancer is still unclear in model of rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide.

Cell proliferation is regarded as one of the most important biological mechanisms in oncogenesis (14). In this context, the expression levels of the Ki-67 nuclear antigen has been linked to prognosis and treatment prediction with varying results in oral cancer and few studies performed exclusively in oral tongue squamous cell carcinoma (OTSCC) (15-19). The expression of Ki-67 has showed similar trends, and increased with the degree of dysplasia of the oral cavity (20). Ki-67 is present throughout the complete cell cycle with the exception of early G1 phase (21). Recently, our group showed that proliferating cellular nuclear antigen (PCNA) is closely involved during neoplastic conversion of squamous cell carcinomas in rat tongue mucosa (8). However, the significance of Ki-67 expression during rat tongue carcinogenesis remains unclear.

Myofibroblasts have an ultrastructural accumulation of alpha-smooth muscle actin (SMA), a contractile apparatus organized as bundles of microfilaments and are found in the stroma and in specialized normal tissue in which they have mechanical functions (21). Stromal collagen deposition by myofibroblasts during cancer invasion is associated with desmoplasia (22) and myofibroblasts may also produce cytokines, such as paracrine

motility factor, hepatocyte growth factor (HGF) (23), or fibroblast growth factor (FGF), which initiate vascular growth (24), increase cancer cell invasiveness, and increase metastatic potential, respectively. Presence of myofibroblasts was distinctively associated in the stroma of oral squamous cell carcinoma (25,26). Considering that angiogenesis plays an important role in tumor growth and metastasis, the relationship between the expression of stromal myofibroblasts and distribution of VEGF in tumor, could be essential to the development and design of new therapeutic strategies, particularly because there are no studies so far.

Thus, the purpose of this study was to investigate if mutations in the genes *H-ras* and *K-ras* were related to mechanism of invasion as a result of the immuno-expression of H-Ras, Ki-67, alpha-SMA and VEGF during 4-nitroquinoline 1-oxide (4NQO)-induced rat tongue carcinogenesis.

Materials and methods

Animals and experimental design

All experimental protocols involving animals conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals. The study was approved by the Animal Committee of Federal University of São Paulo, UNIFESP. Forty male Wistar rats (8 weeks old) weighing approximately 250g were obtained from Centro de Desenvolvimento de Modelos Experimentais (CEDEME), Federal University of Sao Paulo, SP, Brazil. They were maintained under controlled conditions of temperature (24 ±2°C), light-dark periods of 12 h and with free access to water and commercial diet (Nuvital PR, Brazil). The animals were divided into three groups of 10 and were treated with 50 ppm 4NQO (Sigma Aldrich,

St. Louis, USA) solution by drinking water for 4, 12 or 20 weeks. Ten animals were used as control. At the end of the experimental period, the rats were sacrificed by 0.4% sodium pentobarbital (1mL/kg, i.p.). The tongues were longitudinally bisected, half for histopathological examinations and half for gene sequence. For this purpose, half of the tongue were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks, and then stained with hematoxylin and eosin (H.E., Merck) and another half of the tongue was store -80°C, respectively.

Histopathological analysis

Histopathological evaluation was performed by light microscopy. Analysis of the tongue sections were graded as normal, hyperplasia, dysplasia, and carcinoma per animal according to Ribeiro et al. (9).

DNA extraction of fresh tissue

A total of 25mg of the fresh tissue were removed of the areas that corresponding the lesions from 40 rats (10 animals per group) and were placed in tubes. QIAamp DNA Mini Kit (Qiagen) was used to extraction of the DNA according to the manufacturer's protocol and the samples was kept at -20°C.

PCR amplification of the rat H-ras and K-ras genes

PCR primers were chosen to amplify exon 1 and 2 of rat *H-ras* e *K-ras* genes (Table 1). The reaction was performed in a final volume of 30 µL of PCR buffer. PCR products

were performed in GeneAmp PRC System 9700 (Applied Biosystems) and visualized in 2% agarose gels in buffer TBE (Tris-borate 45mM, EDTA 1mM) under UV light, after electrophoresis at 100V/min and gel red staining.

Table1. Oligonucleotide primers and condition used for the exon 1 and 2 of rat *H-Ras* e *K-Ras* genes amplification

Gene	sense	antisense	pb
rHa1	GGCCTGGCTAAGTGTGATTC	TGGGACTCTAACCCATGACC	241
rHa2	AGAGGTAGGACCCTTAAGCTGTG	CTCACGGGCTAGCCATAGGT	240
rKi1	AAGTACTTGATAATCTTGTGTGGAACA	AGGATGACTGCCACCCTTTA	241
rKi2	CCAGACTGTGTTTCTACCTTCTCA	TTAAACCCACCTATAATGGTGAA	214
Conditions	94°C-5 min, 94°C-1 min, 54°C-2 min, 72°C-2 min (409), 72°C-7 min, 4°C-oo		
	Volume used: H_2O x 7.1 + Primer (F 2.3 + R 2.6) + Master Mix x 12 + DNA		

PCR direct sequencing

The PCR products of exon 1 and 2 of rat *H-ras and K-ras* genes were purified using an in-house protocol for PCR products. The PCR products were added to 95% ethanol ice at 4°C at a 1:4 ratio and kept at -20°C for 2 h. The samples were centrifuged (13,000 rpm, 45 min) at room temperature. The supernatant was removed and the pellet was rinsed with 250 µL of 70% ethanol and centrifuged (13,000 rpm, 15 min) at room temperature. The supernatant was removed and the pellet was dried at room temperature. The sample was resuspended in 12 µL of sterile water and maintained at -20°C. Direct sequencing was

performed using PCR primers and ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA) for 25 cycles according to the manufacturer's protocol. The sequence was analyzed using an ABI Prism Genetic Analyzer- 3100 model. The sequencing procedure was performed on both the sense and antisense strands.

Immunohistochemistry

Sections at 3 μm were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol to distilled water. For antigen retrieval slides were placed in 0.01 M citrate-buffer pH 6.0 and heated in a steamer for 30 min. Endogenous peroxidases were quenched by incubating in 3% H_2O_2 for 20 min at room temperature. Sections were incubated overnight at 4 °C with primary antibody: H-Ras mouse monoclonal antibody (1:100), alpha-SMA mouse monoclonal antibody (1:200) and VEGF goat polyclonal antibody (1:200) Santa Cruz Biotechnology, Santa Cruz, CA, USA; Ki-67 mouse monoclonal antibody (1:100), Novocastra Laboratories Ltd, Vision BioSystems Company, Newcastle, United Kingdom. Subsequently, sections were incubated with biotinylated secondary antibody (LSAB, Dakocytomation) for 30 min, washed in PBS, and incubated with streptavidin–peroxidase conjugate (LSAB, Dakocytomation) for 30 min. Finally, the reaction was developed using 3,3′-diaminobenzidine tetrahydrocloride (Sigma) for 5 min. Slides were briefly counterstained in hematoxylin and dehydrated, and cover slips added. Negative and positive controls were to run simultaneously as established in previous studies conduced by our group (7, 27).

Data analysis

Immunostaining was scored by two trained independent observers without prior knowledge of the clinicopathological parameters. Discordant cases were reviewed and agreed upon before data were statistically analyzed. For this purpose, tongue sections stained immunohistochemically were analyzed for the percentages of immunopositive cells in areas diagnosed as normal, hyperplasia, dysplasia, and carcinoma optically. Ki-67 and H-Ras were considered and to the both a total of 1000 epithelial cells were evaluated in three to five fields at 400 magnification. This protocol was established in previous studies conducted by our research group (5). Alpha-SMA was considered to myofibroblasts and VEGF was considered to endothelial cells. A total of 10 fields in the conjunctive tissue were evaluated at x400.

Statistical methods

Statistical analyses for immunohistochemistry data were assessed by one-away ANOVA test followed by post-hoc analysis (Newman-Keuls Multiple Comparison Test) using Graph Pad Prism software pack (version 3.0). A p value <0.05 was considered statistically significant.

Results

Histopathological analysis

Histopathological evaluation following 4NQO treatment showed no histopathological changes in tongue epithelia in the control group (Fig. 1a) or after treatment for 4 weeks

with 4NQO. The primary histopathological change, i.e., hyperplasia and hyperkeratosis with the spinous cell layer gradually thickened, was observed after 12 weeks (Fig.1b). In this period, epithelial dysplasia was also found in mild and moderate forms (Fig. 1c). At 20 weeks, moderate and/or severe oral dysplasia and squamous cell carcinoma (Fig. 1d) were found and the majority of animals had squamous cell carcinoma. The histopathological grade was usually squamous cell carcinoma of a well-differentiated type. The tumors spread into the submucosa and underlying muscle layer, forming small nests with typical keratin pearl formation. In advanced cases, severe atypia was frequently found. The histopathological findings are summarized in the Table 2.

Table 2. Incidence of histopathological lesions in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO)[a] model for oral carcinogenesis.

Groups weeks	N° of animals	Lesions normal	Hyperplasia	Dysplasia	Carcinoma
0	10	10	0	0	0
4	10	10	0	0	0
12	10	0	7	3	0
20	10	0	0	3	7

[a]4NQO, 50 ppm by drinking water

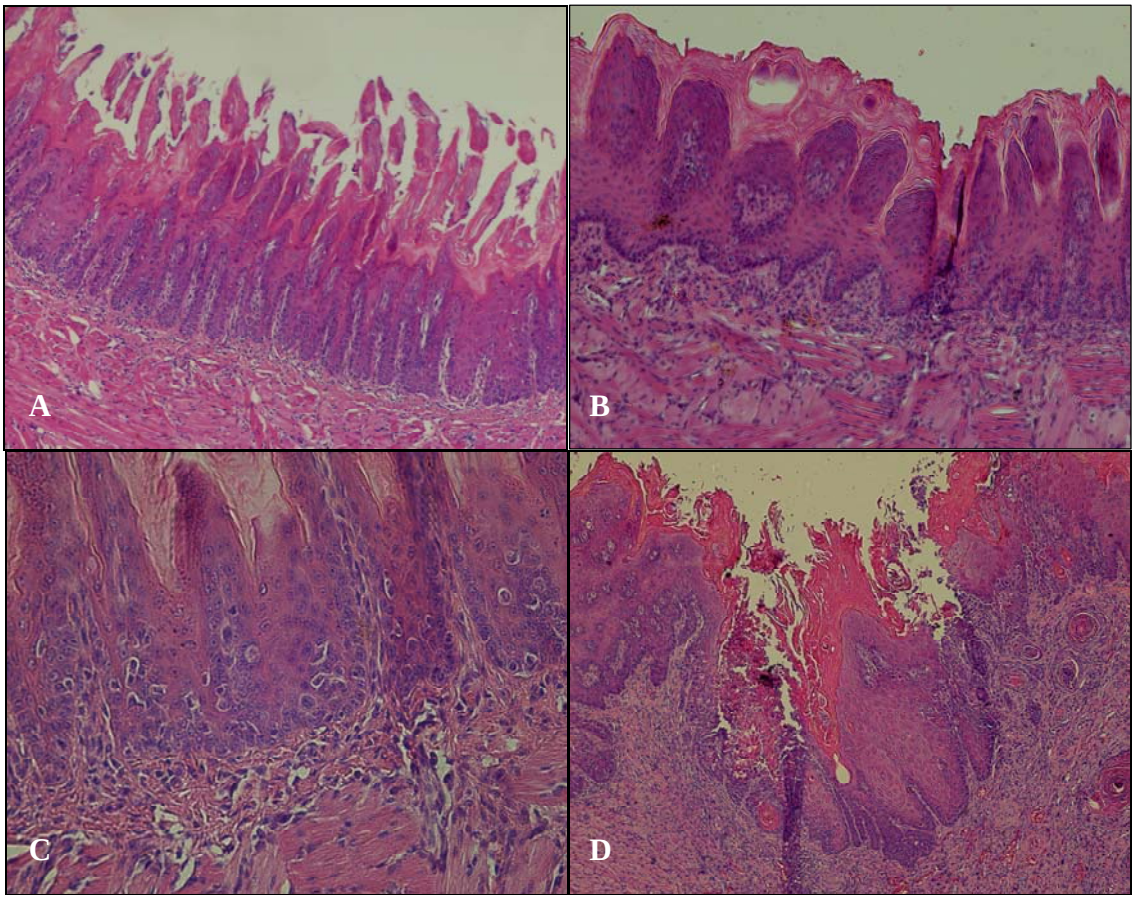

Figure 1. Photomicrographies showing the multi-step process of rat tongue carcinogenesis. (a) No histopathological change (control); (b) hyperplasia and hyperkeratosis (c) epithelial dysplasia; (d) squamous cell carcinoma of well-differentiated type (hematoxylin and eosin stain; 100x magnification).

Mutations in the rat H-ras and K-ras genes

No mutations were detected in any of the experimental periods established in this study, i.e., 4, 12 or 20 weeks of 4NQO treatment corresponding to the 'normal' oral epithelium, hyperplasia, dysplasia and squamous cell carcinomas either to H-ras or K-ras genes (Fig. 2). The same picture occurred in the negative control group, in which no

mutations were detected. Therefore, it was apparently no relationship between histological classification and genomic detection of mutations in this experimental design.

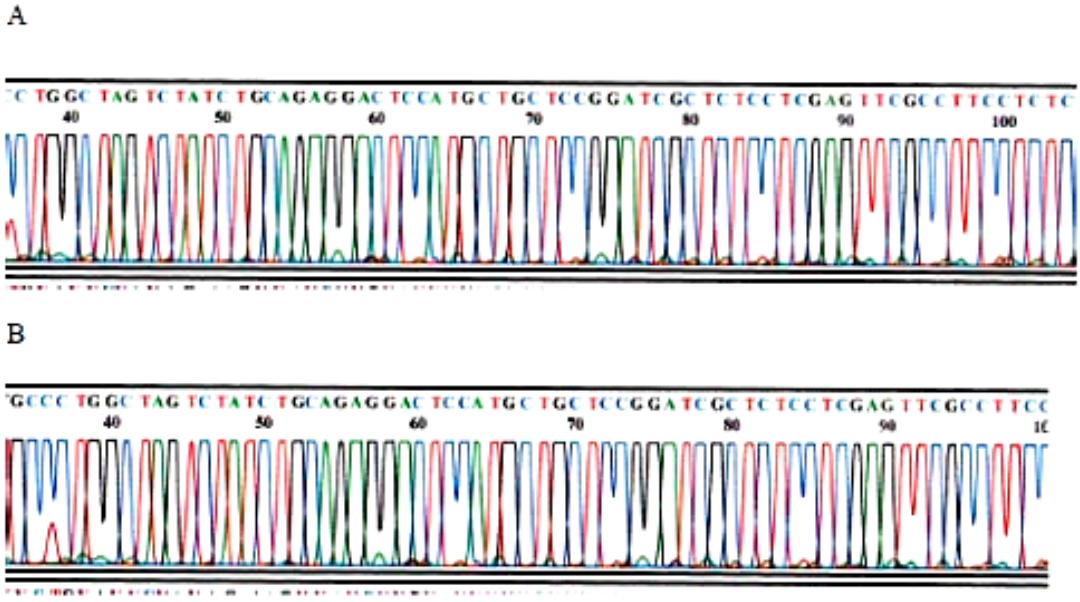

Figure 2. Forward (A) and reverse (B) sequences from *H-ras* exon 1 by direct DNA sequencing method. No mutation was found in any case analyzed.

Immunohistochemistry

The expression of H-Ras was noticed in the cytoplasm and nucleus of oral mucosa cells. Overexpression for this immunomarker were observed for all periods analyzed (Fig. 3). No significant statistically differences ($p>0.05$) was observed between 4, 12 and 20 weeks groups versus the control group. The expression of Ki-67 was detected in the nucleus of oral mucosa cells. In the control group, immunoexpression was present in some cells. However, overexpression was present in 4, 12 and 20 weeks that correspond to the 'normal' oral epithelium, hyperplasia, epithelial dysplasia or neoplastic lesions, respectively

(Fig. 4). Significant statistically differences ($p<0.05$) was observed between 4, 12 and 20 weeks groups versus the control group. These findings are summarized in Fig. 5.

Expression of alpha-SMA was detected in the 20 weeks-treatment only, in the periphery of the tumor's islet on the conjunctive tissue. Specifically for other groups, the expression was closely absent (Fig. 6). Significant statistically differences ($p<0.05$) was observed between 20 weeks group versus the control group. These findings are summarized in Fig. 7. In the same manner, VEGF was observed in endothelial cells following 20 weeks of exposure to carcinogen that correspond to squamous cell carcinomas only (Fig. 8). Since the goal of this study was to show angiogenesis by means VEGF immunoexpression evidenced by tumour (squamous cell carcionoma), we were able to perform the immunohistochemistry after 20 weeks following 4NQO treatment only.

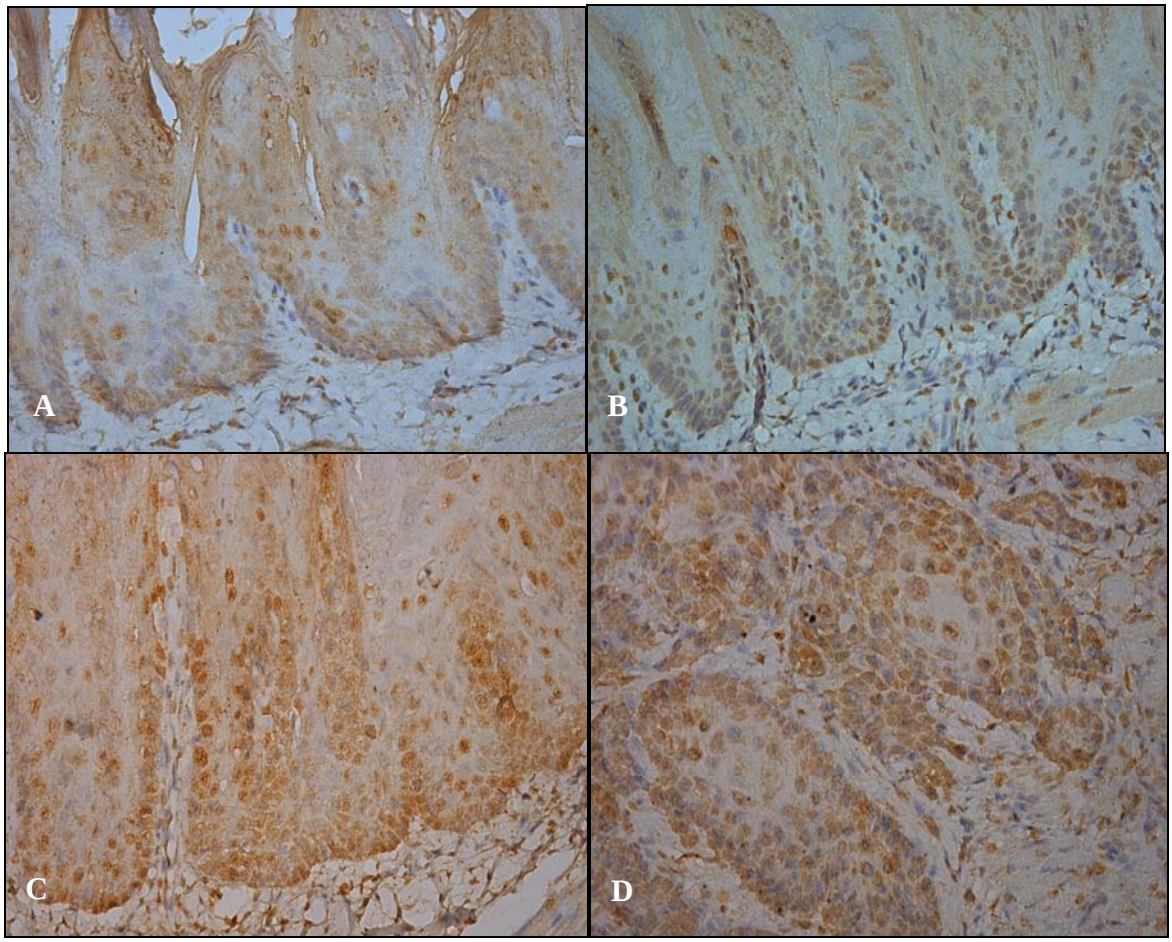

Figure 3. Expression of H-Ras noticed in the nucleus and cytoplasm of oral mucosa cells (a: control; b: 4 weeks; c: 12 weeks; d: 20 weeks; 400x magnification).

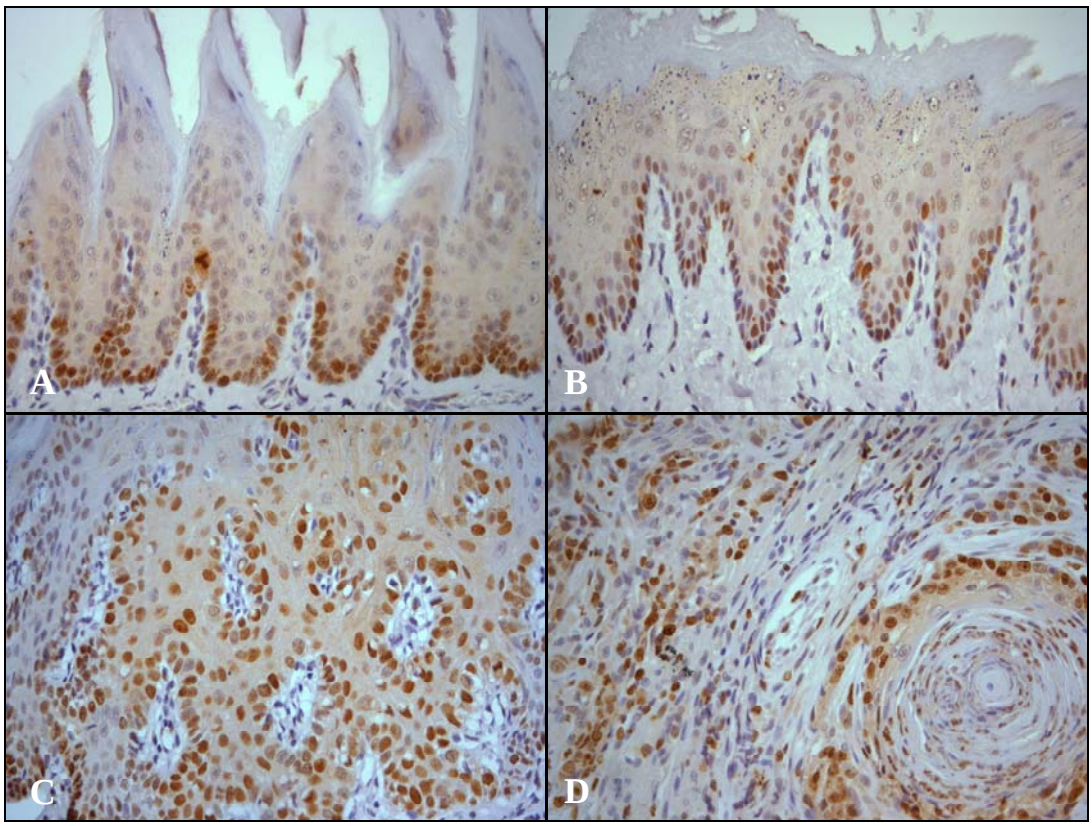

Figure 4. Expression of Ki-67 noticed in the nucleus of oral mucosa cells (a: control; b: 4 weeks; c: 12 weeks; d: 20 weeks; 400x magnification).

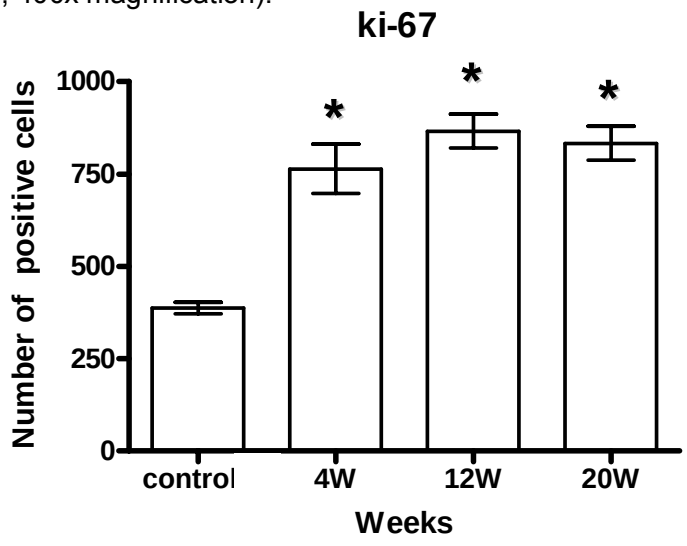

Figure 5. Ki-67 labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 and 20 weeks. Values were expressed as means±S.D. *p<0.05 when compared to negative control group.

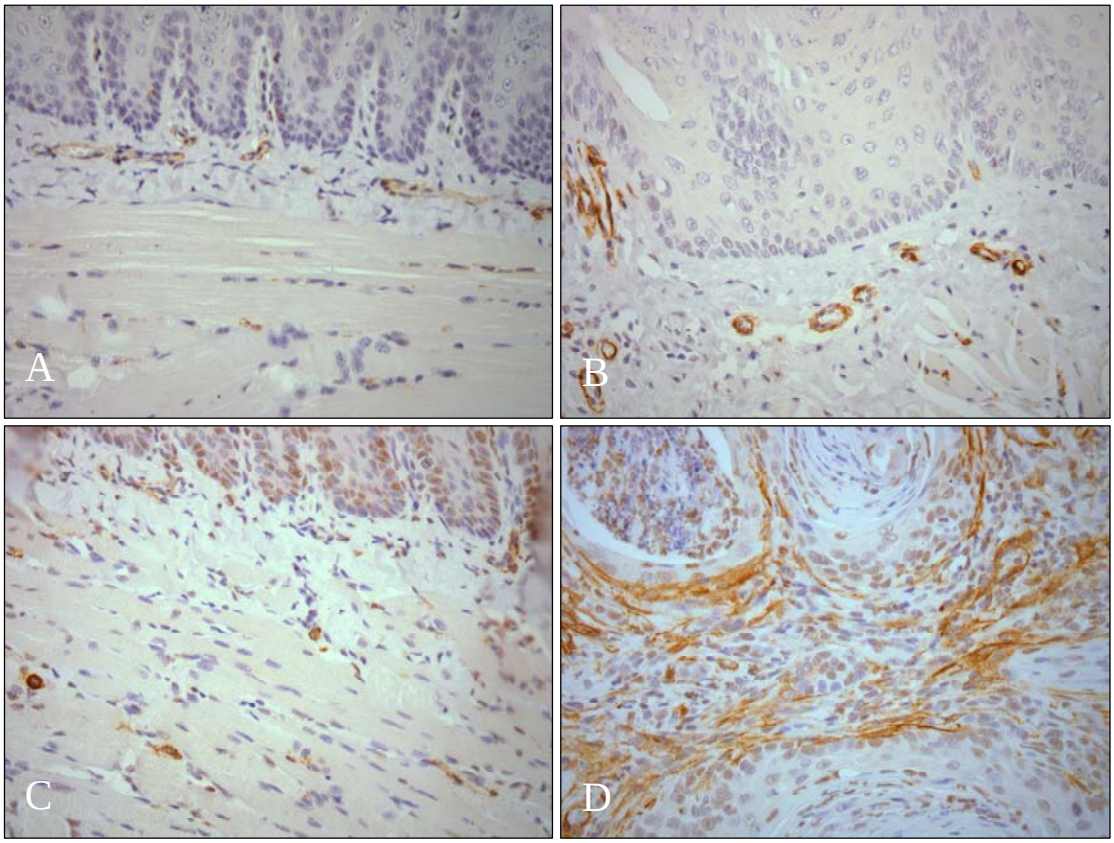

Figure 6. Expression of Alpha-SMA (a: control; b: 4 weeks; c: 12 weeks; d: 20 weeks; 400x magnification). (d) Positive expression of alpha-SMA in myofibroblasts around of the islands in the conjunctive tissue.

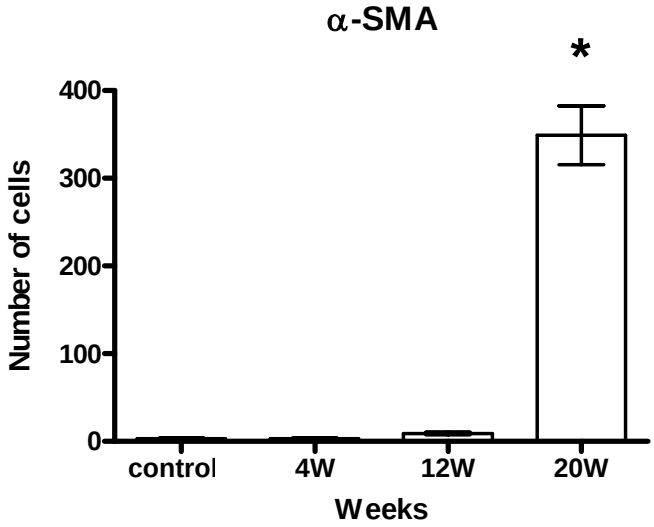

Figure 7. Alpha-SMA to myfibroblasts labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 and 20 weeks. Values were expressed as means ±S.D. *p<0.05 when compared to negative control group.

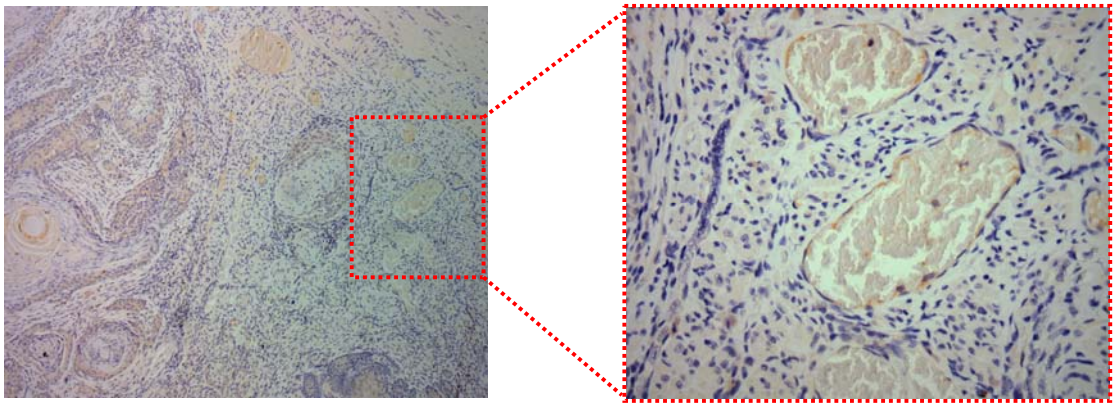

Figure 8. Expression of VEGF in endothelial cells in the 20 weeks; 400X magnification.

Discussion

In this study we investigated mutations in *H-ras* and *K-ras* genes as well as immuno-expression of H-Ras, Ki-67, alpha-SMA and VEGF during experimental oral carcinogenesis using a rat model to establish the significance of several molecular alterations and, thereby, identify risk predictors in oral carcinogenesis (28). To the best of our knowledge, this approach has not been addressed so far.

It has been established that animal models of carcinogenesis allow the isolation of all stages under controlled conditions, including normal tissues, which are then amenable to pathological, genetic, and biochemical analysis, and at lower costs (29). Moreover, chemical carcinogenesis models serve to estimate the risk from environmental agents as well as to determine which precancerous lesions will progress. Several medium term duration assay systems for oral carcinogenesis offer particular promise. Our results, using 4NQO as a carcinogen inducer, demonstrated histopathological changes in rat tongue mucosa along a time-course from hyperplasia, pre-malignant dysplasia, and carcinoma in situ, to invasive squamous cell carcinoma. Therefore, it should be assumed that tongue

carcinogenesis was 4NQO dependent, because these lesions did not occur in the control rats and the rats that developed tumors were younger than 28 weeks of age, when spontaneous tumors are not common in this species (30).

The incidence of mutated *Ras* genes varies strongly among the different tumor types (13). *K-ras* mutations are prevalent in pancreatic, colorectal, endometrial, biliary, lung and cervical cancers (31); *H-ras* mutations predominate in bladder cancer and it has been correlated with oral tumours (32). Suzui et al. (33) found infrequent or absence of mutations in ras genes family following oral carcinogenesis induced by 4-nitroquinoline 1-oxide. In this study, Fischer 344 male rat, an inbred strain was used, being 4NQO applied at a low level dose (20 ppm). Therefore, an evaluation of 4NQO is a necessary step to better establish the medium-term oral carcinogenesis assay with the Wistar strain. Our findings, using a 4NQO as a carcinogen inducer, corroborate with previous findings because no mutations were detected either to *H-ras* or *K-ras* genes for all experimental periods established, i.e., 4, 12 or 20 weeks of 4NQO treatment that corresponding to 'normal' oral mucosa, hyperplasia, dysplasia and squamous cell carcinomas in Wistar rats. It is important to stress that 4NQO is an alkylating compound and potent mutagen that requires metabolic activation to be converted to 4-acetoxyaminoquinoline 1-oxide (4HAQO), which reacts with DNA causing damaging effects including single strand breaks, incomplete repair sites and alkali-labile sites. It has been established that 4HAQO is also able to promote methylation in promoter regions of genes (34,35). This could explain our results. Taken as a whole, it seems that mutations in *H-ras* and *K-ras* genes are not directly involved in the multistage process of rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide.

Ras GTPases are considered cytoplasmic proteins that must be localized to cell membranes for activation. There is some evidence of the presence of Ras isoforms in nuclei of eukaryotic cells (36-38). Wurzer et al. (38) have demmonstrated by immunofluorescence analysis that in addition to its cytosolic localization, activated H-Ras was also localized in the nuclei of transformed cells both in vitro and in vivo. On the occasion, immunoblot analysis of nuclear fractions was consistent with their results found by immunohistochemistry. These findings could emphasize our results, because positive ras immunoexpression was present for all experimental periods adopted in this study indistincly. Probably, the lack of mutation status in the ras genes contributed to the similar expression of ras protein during rat tongue carcinogenesis. This has been confirmed by others (13).

A number of studies have shown that proliferative activity is of high prognostic significance in several types of cancer (39). Expression levels of the Ki-67 nuclear antigen, have showed tendency to increase with the degree of dysplasia of the oral cavity (20). Furthermore, it has been linked to prognosis and treatment prediction in oral cancer (15-19). We found increased levels of expression to Ki-67 in experimental periods that correspond to 4, 12 or 20 weeks of 4NQO treatment compared with the control group. Some authors have revealed increased expression of Ki-67 in dysplastic lesions of mice tongue exposed to 4NQO (40). Furthermore, the labeling index, determined by the numbers of Ki67-positive cells was significantly higher in the dysplastic lesions compared with the nondysplastic epithelium in trangenic mice (40). Using a Wistar outbred strain, our results did not show significant statistically differences from dysplasic lesions and

nondyplastic epithelium. Overall, we assumed that an increase of proliferative status is associated with the risk as well as progression of tongue cancer.

The biological mechanisms of tumorigenesis also results from disturbance between cancer cells and their microenvironment, that consists of host cells (i.e., vascular cells, inflammatory cells, fibroblasts, adipocytes and myoepithelial cells), which, under the influence of and in collaboration with the cancer cells, produce the extra-cellular matrix proteinases and cytokines contributing to tumor invasion and metastasis. Herein, myofibroblasts and vascular endothelial growth are cancer-induced host cells within the tumor microenvironment (41). Our results showed that stromal myofibroblasts were not associated with normal, hyperkeratotic/hyperplasic and dysplasic epithelium. There are some speculations on the source of myfibroblasts in the carcinoma-associated stroma (25). Transforming growth factor beta (TGF-ß) and platelet-derived growth factor (PDGF) are key molecules responsible for the differentiation of fibroblasts, in the wound-healing process (21). TGF-ß and PDGF may also be considered as key cancer-derived molecules that play a role in transdifferentiation and maturation of fibroblasts in their carcinoma-associated stroma (42). A positive corretion between vascular endothelial growth factor (VEGF) and stromal alpha-SMA were detected after 20 weeks of 4NQO treatment in this setting. Chen et al. have revealed that stromal collagen deposition by myofibroblasts during cancer invasion is associated with desmoplasia (22). Myofibroblasts may also produce cytokines, such as paracrine motility factor, hepatocytegrowth factor (HGF) (23), or fibroblast growth factor (FGF), which initiate vascular growth (24), increase cancer cell invasiveness, as far as increase of metastatic potential. Positive VEGF immunoexpression

to microvessel area was noticed to tumor burden. In fact, some studies have found correlation between VEGF expression and angiogenesis in carcinoma (43).

Taken together, our results support the idea that expression of Ki-67 plays a crucial role during malignant transformation being closely related to neoplastic conversion of the oral mucosa cells. It seems that mutations in genes of the ras family did not contribute to pathogenesis of tongue cancer. Further studies are welcomed in human tissues with pre-malignant oral lesions and with oral squamous cell carcinoma since it could be essential to the development and design of new therapeutic strategies.

Acknowledgments

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References

1. Myers JN, Elkins T, Roberts D, Byers RM. Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. Otolaryngol Head Neck Surg 2000; 122:44–51.
2. Kantola S, Parikka M, Jokinen K et al. Prognostic factors in tongue cancer – relative importance of demographic, clinical and histopathological factors. Br J Cancer 2000; 83:614-9.
3. Okazaki Y, Tanaka Y, Tonogi M, Yamane G. Investigation of environmental factors for diagnosing malignant potential in oral epithelial dysplasia. Oral Oncol 2002; 38: 562-73.
4. Vered M, Daniel N, Hirshberg A, Dayan D. Histomorphologic and morphometric changes in minor salivary glands of the rat tongue during 4-nitroquinoline 1-oxide-induced carcinogenesis. Oral Oncol 2003; 39: 491-6.
5. Ribeiro DA, Salvadori DM, Marques ME. Abnormal expression of bcl-2 and bax in rat tongue mucosa during the development of squamous cell carcinoma induced by 4-nitroquinoline 1-oxide. Int J Exp Pathol 2005; 86: 375-81.
6. Niwa, S, Ueno, S, Shirasu, R, 2001. Alteration of pRB expression in the development of rat tongue carcinoma induced by 4-nitroquinoline 1-oxide. Oral Oncol 2001; 37:579-85.
7. Ribeiro DA, Kitakawa D, Domingues MA, Cabral LA, Marques ME, Salvadori DM. Survivin and nitric oxide inducible synthase production during 4NQO-induced rat tongue carcinogenesis, a possible relationship. Exp Mol Pathol 2007; 83: 131-7.
8. Silva RN, Ribeiro DA, Salvadori DM, Marques ME. Placental glutathione S-transferase correlates with cellular proliferation during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Exp Toxicol Pathol 2007; 59: 61-8.
9. Ribeiro DA, Fracalossi AC, Uatari SA, Oshima CT, Salvadori DM. Imbalance of tumor suppression genes expression following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. In Vivo 2009; 23:937-42.
10. Fracalossi AC, Silva MD, Oshima CT, Ribeiro DA. Wnt/beta-catenin signalling pathway following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Exp Mol Pathol 2010; 88:176-83.

11. Naoi K, Sunagawa N, Yoshida I, Morioka T, Nakashima M, Ishihara M, Fukamachi K, Itoh Y, Tsuda H, Yoshimi N, Suzui M. Enhancement of tongue carcinogenesis in Hras128 transgenic rats treated with 4-nitroquinoline 1-oxide. Oncol Rep 2010; 23: 337-44.
12. Chang YS, Yeh KT, Hsu NC, Lin SH, Chang TJ, Chang JG. Detection of N-, H-, and KRAS codons 12, 13, and 61 mutations with universal RAS primer multiplex PCR and N-, H-, and KRAS-specific primer extension. Clin Biochem 2010; 43: 296-301.
13. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res 1989; 49: 4682-9.
14. van Diest PJ, Brugal G, Baak JP. Proliferation markers in tumours: interpretation and clinical value. J Clin Pathol 1998; 51:716-24.
15. Xie X, De Angelis P, Clausen OP, Boysen M. Prognostic significance of proliferative and apoptotic markers in oral tongue squamous cell carcinomas. Oral Oncol 1999; 35: 502 –509.
16. Pich A, Chiusa L, Navone R. Prognostic relevance of cell proliferation in head and neck tumors. Ann Oncol 2004; 15: 319-29.
17. Lothaire P, de Azambuja E, Dequanter D, Lalami Y, Sotiriou C, Andry G, Castro Jr G, Awada A. Molecular markers of head and necksquamous cell carcinoma: promising signs in need of prospective evaluation. Head Neck 2006; 28: 256–69.
18. Kim SJ, Shin HJ, Jung KY, Baek SK, Shin BK, Choi J, Kim BS, Shin SW, Kim YH, Kim JS, Oosterwijk E. Prognostic value of carbonic anhydrase IX and Ki-67 expression in squamous cell carcinoma of the tongue. Jpn J Clin Oncol 2007; 37: 812–9.
19. Wangsa D, Ryott M, Avall-Lundqvist E, Petersson F, Elmberger G, Luo J, Ried T, Auer G, Munck-Wikland E. Ki-67 expression predicts locoregional recurrence in stage I oral tongue carcinoma. Br J Cancer 2008; 99:1121-1128.
20. Angiero F, Berenzi A, Benetti A, Rossi E, Del Sordo R, Sidoni A, Stefani M, Dessy E. Expression of p16, p53 and Ki-67 proteins in the progression of epithelial dysplasia of the oral cavity. Anticancer Res 2008; 28: 2535-39.
21. Tomasek JJ, Gabbiani G, Hinz B, Chaponnien C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodeling. Nat Rev Mol Cell Biol 2002; 3:349-63.

22. Chen Y, Satoh T, Sasatomi E, Miyazaki K, Tokunaga O. Critical role of type IV collagens in the growth of bile duct carcinoma. Pathol Res Pract 2001; 197: 585-96.
23. Lewis MP, Lygoe KA, Nystrom ML, et al. Tumor-derived TGF-b1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. Br J Cancer 2004; 90: 822-32.
24. Rousseau B, Larrieu-Lahargue F, Javerzat S, Guilhem-Ducleon F, Beermann F, Bikfalvi A. The tryp1-Tag/tryp1-FGFR-DN biogenic mouse: a model for selective inhibition of tumor development, angiogenesis, and invasion into the neural tissue by blocking fibroblast growth factor receptor activity. Cancer Res 2004; 64: 2490-5.
25. Vered M, Allon I, Buchner A, Dayan D. Stromal myofibroblasts and malignant transformation in a 4NQO rat tongue carcinogenesis model. Oral Oncol 2007; 43:999-1006.
26. Etemad-Moghadam S, Khalili M, Tirgary F, Alaeddini M. Evaluation of myofibroblasts in oral epithelial dysplasia and squamous cell carcinoma. J Oral Pathol Med 2009; 38: 639-43.
27. Badiglian Filho L, Oshima CT, De Oliveira Lima F, De Oliveira Costa H, De Sousa Damião R, Gomes TS, Gonçalves WJ. Canonical and noncanonical Wnt pathway: a comparison among normal ovary, benign ovarian tumor and ovarian cancer. Oncol Rep 2009; 21:313-20.
28. Shah NG, Trivedi TI, Tankshali RA, Goswami, J.A., Shah, J.S., Jetly, D.H., Kobawala, T.P., Patel, K.C., Shukla, S.N., Shah, P.M., Verma, R.J. Molecular alterations in oral carcinogenesis, significant risk predictors in malignant transformation and tumor progression. Int. J. Biol. Markers 2007; 22: 132-43.
29. Herzig, M, Christofori, G. Recent advances in cancer research, mouse models of tumorigenesis. Biochim Biophys Acta 2002; 1602: 97-113.
30. Hayashi, S, Nonoyama, T, Miyajima, H. Spontaneous nonthymic cell lymphomas in young Wistar rats. Vet Pathol 1989; 26: 326-32.
31. Minamoto T, Mai M, Ronai Z. K-ras mutation: early detection in molecular diagnosis and risk assessment of colorectal, pancreas, and lung cancers--a review. Cancer Detect Prev 2000; 24: 1-12.

32. Hoellering J, Shuler CF. Localization of H-ras mRNA in oral squamous cell carcinomas. J Oral Pathol Med 1989; 18: 74-8.
33. Suzui, M, Yoshimi, N, Tanaka, T, Mori, H. Infrequent Ha-ras mutations and absence of Ki-ras, N-ras, and p53 mutations in 4-nitroquinoline 1-oxide-induced rat oral lesions. Mol Carcinog 1995; 14: 294-8.
34. Nakahara Y, Shintani S, Mihara M, Matsumura T, Hamakawa H. High frequency methylation of p16INK4A gene during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Oncol Rep 2004; 12:101–6.
35. Ogawa K, Tanuma J, Hirano M, Hirayama Y, Semba I, Shisa H, Kitano M. Selective loss of resistant alleles at p15INK4B and p16INK4A genes in chemically-induced rat tongue cancers. Oral Oncol 2006; 42:710–7.
36. Fuentes-Calvo I, Blázquez-Medela AM, Santos E, López-Novoa JM, Martínez-Salgado C. Analysis of k-ras nuclear expression in fibroblasts and mesangial cells. PLoS One. 2010; 14;5:e8703.
37. Birchenall-Roberts MC, Fu T, Kim SG, Huang YK, Dambach M, et al. K-Ras4B proteins are expressed in the nucleolus: Interaction with nucleolin. 2006; Biochem Biophys Res Commun 348: 540-9.
38. Wurzer G, Mosgoeller W, Chabicovsky M, Cerni C, Wesierska-Gadek J. Nuclear Ras: unexpected subcellular distribution of oncogenic forms, J Cell Biochem 2001; 81:1-11.
39. Tubiana M, Courdi A. Cell proliferation kinetics in human solid tumors: relation to probability of metastatic dissemination and long-term survival. Radiother Oncol 1989; 15: 1-18.
40. Wilkey JF, Buchberger G, Saucier K, Patel SM, Eisenberg E, Nakagawa H, Michaylira CZ, Rustgi AK, Mallya SM. Cyclin D1 overexpression increases susceptibility to 4-nitroquinoline-1-oxide-induced dysplasia and neoplasia in murine squamous oral epithelium. Mol Carcinog 2009;48:853-61.
41. Desmouliere A, Guyot C, Gabbiani G. The stroma reaction myofibroblasts: a key player in the control of tumor behavior. Int J Dev Biol 2004; 48:509-17.
42. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. J Pathol 2003; 200: 429-47.

43. Mărgăritescu C, Pirici D, Simionescu C, Mogoantă L, Raica M, Stîngă A, Ciurea R, Stepan A, Stîngă A, Ribatti D. VEGF and VEGFRs expression in oral squamous cell carcinoma. Rom J Morphol Embryol 2009; 50:527-48.

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ORIGINAL PAPER

The role of matrix metalloproteinases 2 and 9 during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide

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Abstract Matrix metalloproteinases (MMPs) are implicated in a wide range of physiological and pathological processes, including morphogenesis, wound healing, angiogenesis, inflammation, and cancer. The purpose of this study was to characterize the role of MMPs as depicted by the expression of MMP-2 and MMP-9 during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Male Wistar rats were distributed into three groups of 10 animals each and treated with 4-nitroquinoline 1-oxide solution at 50 ppm through their drinking water for 4, 12, and 20 weeks. Ten animals were used as control group. No histopathological abnormalities were induced in the epithelium after 4 weeks of carcinogen exposure; however, immunoexpression of MMP-2 was noticed. The same picture occurred to MMP-9, in which positive expression was detected for this immunomarker. MMP-2 and MMP-9 showed positive expression either in pre-neoplastic lesions at 12 weeks following carcinogen exposure or in well-differentiated squamous cell carcinoma induced after 20 weeks of treatment with 4NQO. Taken together, our results support the belief that MMP-2 and MMP-9 play important role during malignant transformation and conversion of oral mucosa as assessed by immunohistochemistry.

Keywords Oral squamous cell carcinoma · 4-nitroquinoline 1-oxide · Immunohistochemistry · Matrix metalloproteinase

Introduction

The matrix metalloproteinases (MMPs) are a large group of zinc-dependent enzymes responsible for degradation of extracellular matrix components, including collagen, gelatin, fibronectin, laminin and proteoglycan in normal physiological and pathologic processes such as embryogenesis, wound healing, angiogenesis, tissue remodeling, tumor invasion and metastasis (Coussens et al. 2002). The MMP-2, also designated type IV collagenase, cleaves collagen types IV, V, VII, X and gelatin type I, whereas MMP-9, also designated 92-kDa type IV collagenase or gelatinase B, has been shown to degrade bone collagen. On the other context, theses enzymes are also involved in the progression and metastasis of several tumors (Deryugina and Quigley 2010).

Particularly, studies have commonly identified MMP-2 and MMP-9 associated with lymph node metastasis and poor outcome in laryngeal cancer (Xie et al. 2004). Otherwise, immunoexpression of these proteins failed to demmonstrate the relationship with tumor invasion, size and tumor growth in esophageal squamous cell carcinoma (Lukaszewicz-Zajac et al. 2009). Microarray gene expression studies on whole in head and neck squamous cell carcinoma (HNSCC) tumor samples have identified overexpression of MMP-2 (Chung et al. 2004). Increased active MMP-2 and MMP-9 has prognostic importance in

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squamous cell carcinomas of the oral cavity (Yorioka et al. 2002). High expression levels of MMP-2 and MMP-9 has been shown during malignant transformation in the oral mucosa (Bindhu et al. 2006).

Oral squamous cell carcinoma is the most common head and neck cancer and it often has a poor prognosis, owing to local tumor invasion and frequent lymph node metastasis (Arenas-Huertero et al. 1999). The development of oral cancer is usually preceded by a premalignant phase, the most common of which is leukoplakia. Malignant transformation of these leukoplakias varies in different parts of the world, probably as a result of differences in tobacco and dietary habits, which ranges from 3% to 8.1% (Scheifele and Reichart 2003). Although epithelial dysplasia is an important predictive factor of malignant transformation, not all dysplastic lesions will become malignant. The mechanisms involved in the transformation of premalignant/potentially malignant lesions to invasive cancer are still largely unknown. Probably, perturbations in the production, deposition, and degradation of the extracellular matrix (ECM) occur during neoplastic transformation and further progression events contributing to tumorigenesis.

A good experimental model for studying oral carcinogenesis is by water-soluble 4-nitroquinoline 1-oxide (4NQO) (Ribeiro et al. 2007). The medium-term assay is based on the multi-step process of carcinogenesis characterized by initiation, promotion and tumor progression. Chronic administration of 4NQO in drinking water simulates rat tongue carcinogenesis like human counterpart (Ribeiro et al. 2004, 2005, 2007). In this regard, it would be useful to know if, and to what extent MMPs 2 and 9 are involded during oral carcinogenesis, particularly because there are no previous reports.

As a result and because the lack of scientifc evidence, the aim of this study was to investigate the expression of MMP-2 and MMP-9 following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide.

Materials and methods

Animals and experimental design

All experimental protocols involving animals conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals. The study was approved by the Animal Committee of Federal University of Sao Paulo, UNIFESP.

Forty male Wistar rats (8 weeks old) weighing approximately 250 g, were obtained from Centro de Desenvolvimento de Modelos Experimentais (CEDEME), Federal University of Sao Paulo, SP, Brazil. They were maintained under controlled conditions of temperature (24 ± 2°C), light–dark periods of 12 h, and with free access to water and commercial diet (Nuvital PR, Brazil). The animals were divided into 3 groups of 10 and were treated with 50 ppm 4NQO (Sigma–Aldrich, St. Louis, USA) solution by drinking water for 4, 12 or 20 weeks. All rats were allowed access to the drinking water, which was replaced twice a week with freshly prepared solution. Bottles of drinking water were shielded with aluminum foil. Ten animals were used as control. At the end of the experimental period, the rats were sacrificed by 0.4% sodium pentobarbital (1 ml/kg, i.p.). The tongues were longitudinally bisected for histopathological examinations. The tissues were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks, and stained with hematoxylin and eosin (H.E., Merck).

Histopathological analysis

Histopathological evaluation was performed by light microscopy. Analyzes of the tongue sections were graded as normal, hyperplasia, dysplasia, and carcinoma per animal according to Ribeiro et al. (2007).

Immunohistochemistry

Sections at 3 μm were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol to distilled water. For antigen retrieval slides were placed in 0.01 M citrate-buffer pH 6.0 and heated in a steamer for 30 min. Endogenous peroxidases were quenched by incubating in 3% H_2O_2 for 20 min at room temperature. Sections were incubated overnight at 4°C with primary antibody: MMP-2 mouse monoclonal anti-rat antibody (1:50) (8B4) and MMP-9 mouse monoclonal anti-rat antibody (1:100) (2C3), Santa Cruz Biotechnology, Santa Cruz, CA, USA. Subsequently, sections were incubated with biotinylated secondary antibody (LSAB, Dakocytomation) for 30 min, washed in PBS, and incubated with streptavidin-peroxidase conjugate (LSAB, Dakocytomation) for 30 min. Finally, the reaction was developed using 3,3′-Diaminobenzidine tetrahydrocloride (Sigma) for 5 min. Slides were briefly counterstained in hematoxylin and dehydrated, and cover slips added. Negative and positive controls were to run simultaneously. Positive controls were represented by mammary tissue. Negative controls were made by eliminating the primary antibody as established in previous studies conduced by our group (Ribeiro et al. 2007; Badiglian Filho et al. 2009).

Data analysis

Immunostaining was scored by two trained independent observers (ACCF and SRM) without prior knowledge of

the clinicopathological parameters. Discordant cases were reviewed and agreed upon before data were statistically analyzed. For this purpose, tongue sections stained immunohistochemically were analyzed for the percentages of immunopositive cells in areas diagnosed as normal, hyperplasia, dysplasia, and carcinoma optically. A total of 1,000 epithelial cells were evaluated in 3–5 fields at ×400 magnification. All values were used as labeling indices. This protocol was established in previous studies conducted by our research group (Ribeiro et al. 2005).

Statistical methods

Statistical analyses for immunohistochemistry data were assessed by Kruskal–Wallis non-parametric test followed by post-hoc analysis (Dunn's test) using SPSS software pack (version 1.0). A P value < 0.05 was considered statistically significant.

Results

Histopathological evaluation following 4NQO treatment

No histopathological changes in tongue epithelia were observed in the control group (Fig. 1a) or after treatment for 4-weeks with 4NQO. The primary histopathological change, i.e., hyperplasia and hyperkeratosis with the spinous cell layer gradually thickened, was observed after 12-weeks (Fig. 1b). In this period, epithelial dysplasia was also found in mild and moderate forms (Fig. 1b). At 20 weeks, moderate and/or severe oral dysplasia and squamous cell carcinoma (Fig. 1c) were found; the majority of animals had squamous cell carcinoma. The histopathological grade was usually squamous cell carcinoma of a well-differentiated type. The tumors spread into the submucosa and underlying muscle layer, forming small

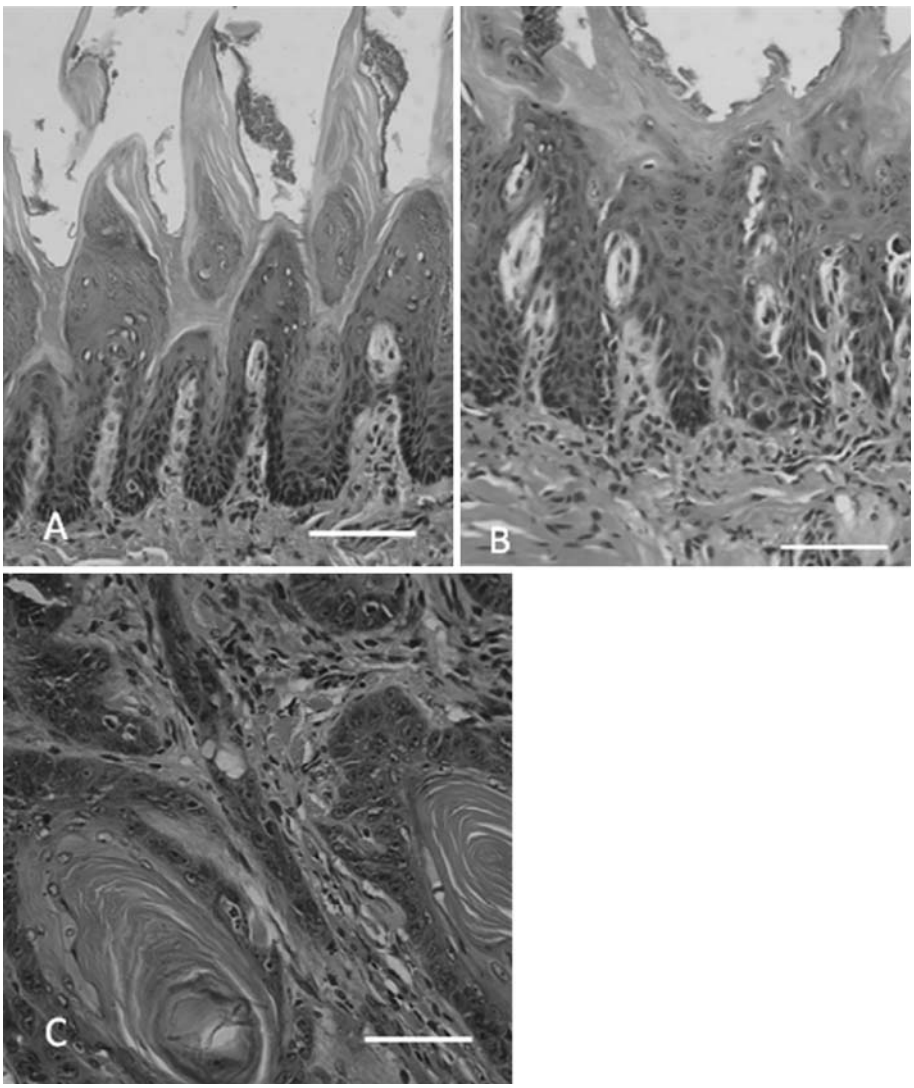

Fig. 1 Photomicrographies showing the multi-step process of rat tongue carcinogenesis. **a** no histopathological change (control); **b** epithelial dysplasia; **c** squamous cell carcinoma of well-differentiated type. (Hematoxylin and Eosin stain; *Bar* = 18 μm)

Table 1 Incidence of histopathological lesions in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO) model for oral carcinogenesis

4NQO–50 ppm by drinking water

Groups (week)	No. of animals	Lesions			
		Normal	Hyperplasia	Dysplasia	Carcinoma
0 (Control)	10	10	0	0	0
4	10	10	0	0	0
12	10	0	7	3	0
20	10	0	0	3	7

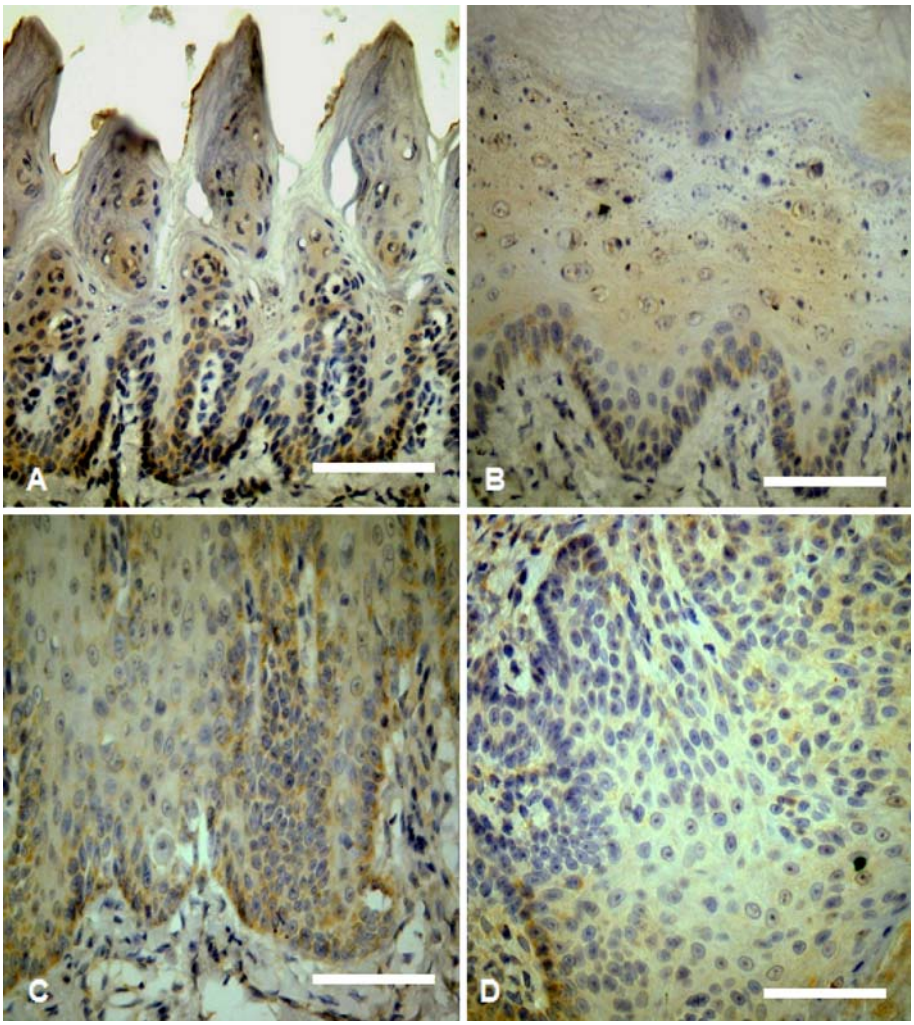

Fig. 2 Expression of MMP-2 noticed in cytoplasm of oral mucosa cells. (**a**: control; **b**: 4 weeks; **c**: 12 weeks; **d**: 20 weeks; *Bar* = 26 μm)

nests with typical keratin pearl formation. In advanced cases, severe atypia was frequently found. The histopathological findings are summarized in the Table 1.

Immunohistochemistry

The expression of MMP-2 was detected in the cytoplasm of oral mucosa cells. In the control group, immunoexpression was detected in all layers of epithelium indistinclty (Fig. 2a). In the group exposed to 4NQO during 4 weeks, a positive expression was detected in oral mucosa cells as well (Fig. 2b). The MMP-2 expression was also present in the majority of animals for 12 and 20 weeks that corresponds to epithelial dysplasia or neoplastic lesions, respectively (Fig. 2c, d, respectively). No significant statistically differences ($P > 0.05$) were observed between 4, 12 and 20 weeks groups versus the control group. These findings are summarized in Fig. 3.

Indistinct expression of MMP-9 was found in the cytoplasm. In the control group and animals exposed to 4NQO for 4 weeks, positive expression was detected (Fig. 4a, b, respectively). In this period, no significant statistically

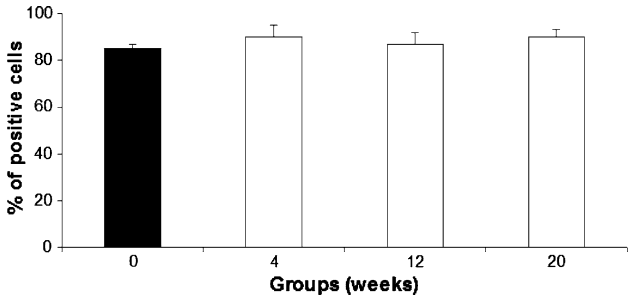

Fig. 3 MMP-2 labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 and 20 weeks. Values were expressed as means ± S.D. NS

differences ($P > 0{,}05$) arose when compared to the control group. Dysplastic lesions and well-differentiated squamous cell carcinomas had positive expression of MMP-9 without any significant differences when compared to negative control ($P > 0{,}05$) (Fig. 4c, d, respectively). All numerical data are summarized in Fig. 5.

Discussion

The aim of this study was to characterize the behavior of MMPs during experimental oral carcinogenesis using a rat model to establish the significance of several molecular alterations and, thereby, identify risk predictors in oral carcinogenesis (Shah et al. 2007). To the best of our knowledge, this approach is new.

It has been established that animal models of carcinogenesis allow the isolation of all stages under controlled conditions, including normal tissues, which are then amenable to pathological, genetic, and biochemical analysis, and at lower costs (Herzig and Christofori 2002). Moreover, chemical carcinogenesis models serve to estimate the risk from environmental agents as well as to determine which precancerous lesions will progress. Several medium term duration assay systems for oral carcinogenesis offer particular promise. Our results, using 4NQO as a carcinogen inducer, demonstrated histopathological changes in rat

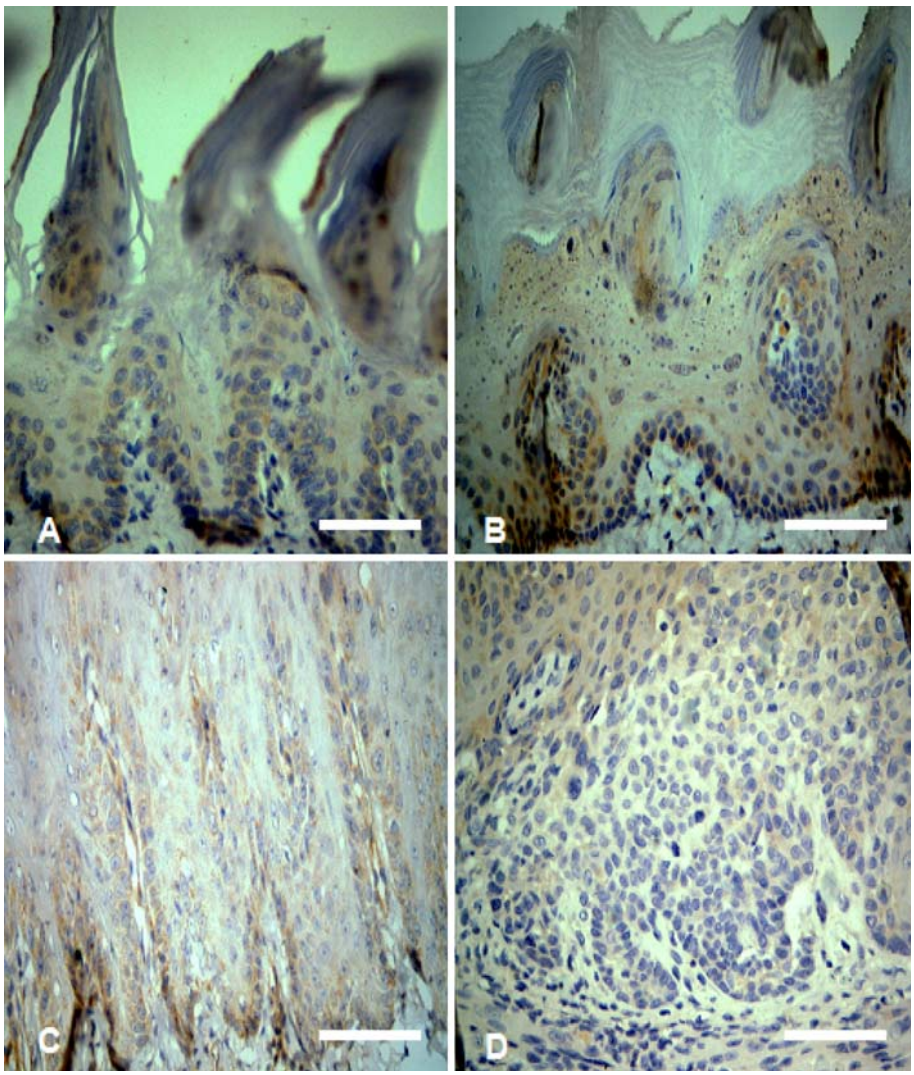

Fig. 4 Expression of MMP-9 noticed in cytoplasm of oral mucosa cells. (**a** control; **b** 4 weeks; **c** 12 weeks; **d** 20 weeks; *Bar* = 24 μm)

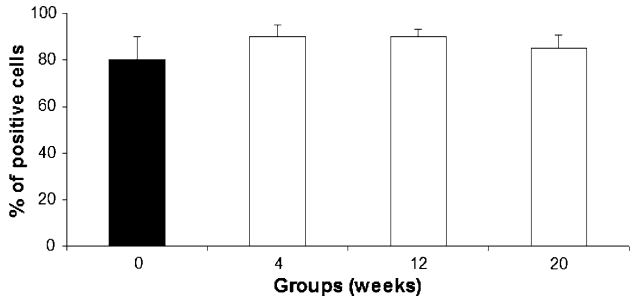

Fig. 5 MMP-9 labeling index in the negative control (zero) and those exposed to 4-nitroquinoline 1-oxide for 4, 12 and 20 weeks. Values were expressed as means ± S.D. NS

tongue mucosa along a time-course from hyperplasia, premalignant dysplasia, and carcinoma in situ, to invasive squamous cell carcinoma. Therefore, it should be assumed that tongue carcinogenesis was 4NQO dependent, because these lesions did not occur in the control rats and the rats that developed tumors were younger than 28 weeks of age, when spontaneous tumors are not common in this species (Hayashi et al. 1989). Previous studies conducted by our group have demmonstrated that Wistar rats are particularly sensitive animals to 4NQO for inducing oral cancer (Minicucci et al. 2009; Ribeiro et al. 2008; Silva et al. 2007).

It is well known that matrix metalloproteinases (MMPs) are an important group of proteolytic enzymes that are capable of degrading the basement membrane, as well as certain cell membrane proteins. They are classified into collagenases, gelatinases, stromelysins and membrane type (MT)-MMPs based on their structure and substrate specificity (Li et al. 1998). Type IV collagenases, MMP2 and MMP9, degrade connective tissue and basement membrane collagen and have been associated with tumor angiogenesis (Stetler-Stevenson and Yu 2001). One purpose of the current study was to clarify whether MMPs are involved in the initial stages of oral carcinogenesis induced by 4NQO. We found that MMP-2 and -9 were present in oral mucosa cells after 4 weeks-treatment, suggesting therefore, MMP-2 and MMP-9 are present during neoplastic transformation of oral mucosa cells.

Although MMP-2 and MMP-9 are almost identical proteinases, their contribution to biological or pathological processes can be very different and at present it is still unclear which one is more important in tumour progression and metastasis. Some studies have indicated that MMP-2 is especially important in the degradation of the extracellular matrix associated with invasion and metastasis of carcinoma cells and is an important determinant of cancer cell behavior (Xu et al. 2005). In addition, MMP- 9 (gelatinase B) has an important role in tumor growth, invasion and metastasis (Nelson et al. 2000). In present study, we also found high positive staining (90–100%) of MMP-2 in the all layers from moderate and/or severe oral dysplasia and squamous cell carcinomas. Such findings are in agreement with previous studies in human (Vilen et al. 2008; Shang et al. 2008).

The MMP-9 was also expressed in the cytoplasm of majority of animals for the 12 or 20 weeks following treatment with 4NQO. A direct correlation between enhanced MMP expression and the invasive phenotype of malignant tumors has been extensively discussed in previous studies (Tsai et al. 2003). They are believed to play an important role in skin and uveal melanoma progression (Seftor et al. 2001), and are associated with poor clinical outcome in various malignancies such as bladder, breast, lung cancer, and head and neck squamous cell carcinomas (Dunne et al. 2003). Our data are fully in line with these findings.

Taken as a whole, our results suggest that the expression of MMPs 2 and 9 play important role during oral carcinogenesis. Applicability to clinical practice for persons at high risk of oral cancer, such as in smokers or in alcoholism, as well as patients diagnosed with oral dysplasia or carcinoma, remains to be developed.

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References

Arenas-Huertero FJ, Herrera-Goepfert R, Delgado-Chavez R, Zinser-Sierra JW, De la Garza-Salazar JG, Herrera-Gomez A, Perez-Cardenas E (1999) Matrix metalloproteinases expressed in squamous cell carcinoma of the oral cavity: correlation with clinicopathologic features and neo-adjuvant chemotherapy response. J Exp Clin Cancer Res 18:279–284

Badiglian Filho L, Oshima CT, De Oliveira Lima F, De Oliveira Costa H, De Sousa Damião R, Gomes TS, Gonçalves WJ (2009) Canonical and noncanonical Wnt pathway: a comparison among normal ovary, benign ovarian tumor and ovarian cancer. Oncol Rep 21:313–320

Bindhu OS, Ramadas K, Sebastian P, Pillai MR (2006) High expression levels of nuclear factor kappa B and gelatinases in the tumorigenesis of oral squamous cell carcinoma. Head Neck 28:916–925

Chung AW, Radomski A, Alonso-Escolano D, Jurasz P, Stewart MW, Malinski T, Radomski MW (2004) Platelet-leukocyte aggregation induced by PAR agonists: regulation by nitric oxide and matrix metalloproteinases. Br J Pharmacol 143:845–855

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 295:2387–2392

Deryugina EI, Quigley JP (2010) Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: Contrasting, overlapping and compensatory functions. Biochim Biophys Acta 1803:103–120

Dunne AA, Sesterhenn A, Gerisch A, Teymoortash A, Kuropkat C, Werner JA (2003) Expression of MMP-2, -9 and -13 in cell lines and fresh biopsies of squamous cell carcinomas of the upper aerodigestive tract. Anticancer Res 23:2233–2240

Hayashi S, Nonoyama T, Miyajima H (1989) Spontaneous nonthymic cell lymphomas in young Wistar rats. Vet Pathol 26:326–332

Herzig M, Christofori G (2002) Recent advances in cancer research, mouse models of tumorigenesis. Biochim Biophys Acta 1602:97–113

Li H, Bauzon DE, Xu X, Tschesche H, Cao J, Sang QA (1998) Immunological characterization of cell-surface and soluble forms of membrane type I matrix metalloproteinase in human breast cancer cells and in fibroblasts. Mol Carcinog 22:84–89

Lukaszewicz-Zajac M, Mroczko B, Kozłowski M, Nikliński J, Laudański J, Szmitkowski M (2009) Elevated levels of serum metalloproteinase 9 in patients with esophageal squamous cell carcinoma. Pol Arch Med Wewn 119:558–563

Minicucci EM, da Silva GN, Ribeiro DA, Favero Salvadori DM (2009) No mutations found in exon 2 of gene p16CDKN2A during rat tongue carcinogenesis induced by 4-nitroquinoline-1-oxide. J Mol Histol 40:71–76

Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM (2000) Matrix metalloproteinases: biologic activity and clinical implications. J Clin Oncol 18:1135–1149

Ribeiro DA, Fávero Salvadori DM, da Silva RN, Ribeiro Darros B, Alencar Marques ME (2004) Genomic instability in non-neoplastic oral mucosa cells can predict risk during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Oral Oncol 40:910–915

Ribeiro DA, Salvadori DM, Marques ME (2005) Abnormal expression of bcl-2 and bax in rat tongue mucosa during the development of squamous cell carcinoma induced by 4-nitroquinoline 1-oxide. Int J Exp Pathol 86:375–381

Ribeiro DA, Kitakawa D, Domingues MA, Cabral LA, Marques ME (2007) Survivin and inducible nitric oxide synthase production during 4NQO-induced rat tongue carcinogenesis: a possible relationship. Exp Mol Pathol 83:131–137

Ribeiro DA, Grilli DG, Salvadori DM (2008) Genomic instability in blood cells is able to predict the oral cancer risk: an experimental study in rats. J Mol Histol 39:481–486

Scheifele C, Reichart PA (2003) Is there a natural limit of the transformation rate of oral leukoplakia? Oral Oncol 39:470–475

Seftor REB, Seftor EA, Koshikawa N (2001) Cooperative interaction of laminin 5 γ2 chain, matrix metalloproteinase-2, and membrane type-1-matrix metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. Cancer Res 61:6322–6327

Shah NG, Trivedi TI, Tankshali RA, Goswami JA, Shah JS, Jetly DH, Kobawala TP, Patel KC, Shukla SN, Shah PM, Verma RJ (2007) Molecular alterations in oral carcinogenesis, significant risk predictors in malignant transformation and tumor progression. Int J Biol Markers 22:132–143

Shang ZJ, Ethunandan M, Górecki DC, Brennan PA (2008) Aberrant expression of beta-dystroglycan may be due to processing by matrix metalloproteinases-2 and -9 in oral squamous cell carcinoma. Oral Oncol 44:1139–1146

Silva RN, Ribeiro DA, Salvadori DM, Marques ME (2007) Placental glutathione S-transferase correlates with cellular proliferation during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Exp Toxicol Pathol 59:61–68

Stetler-Stevenson WG, Yu AE (2001) Proteases in invasion: matrix metalloproteinases. Semin Cancer Biol 11:143–152

Tsai CH, Hsieh YS, Yang SF, Chou MY, Chao ChangY (2003) Matrix metalloproteinase 2 and matrix metalloproteinase 9 expression in human oral squamous cell carcinoma and the effect of protein kinase C inhibitors: preliminary. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 95:710–716

Vilen ST, Nyberg P, Hukkanen M, Sutinen M, Ylipalosaari M, Bjartell A, Paju A, Haaparanta V, Stenman UH, Sorsa T, Salo T (2008) Intracellular co-localization of trypsin-2 and matrix metalloprotease-9: possible proteolytic cascade of trypsin-2, MMP-9 and enterokinase in carcinoma. Exp Cell Res 314: 914–926

Xie M, Sun Y, Li Y (2004) Expression of matrix metalloproteinases in supraglottic carcinoma and its clinical implication for estimating lymph node metastases. Laryngoscope 114:2243–2248

Xu X, Wang Y, Chen Z, Sternlicht MD, Hidalgo M, Steffensen B (2005) Matrix metalloproteinase-2 contributes to cancer cell migration on collagen. Cancer Res 65:130–136

Yorioka CW, Coletta RD, Alves F, Nishimoto IN, Kowalski LP, Graner E (2002) Matrix metalloproteinase-2 and -9 activities correlate with the disease-free survival of oral squamous cell carcinoma patients. Int J Oncol 20:189–194

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Wnt/β-catenin signalling pathway following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide

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ABSTRACT

The Wnt/β-catenin signaling pathway plays an important role in development, tissue homeostasis, and regeneration. Inappropriate activation of the Wnt pathway is linked to a wide range of human cancers. The purpose of this study was to characterize the Wnt/β-catenin signaling pathway as depicted by the expression of Wnt1, Frizzled-1, Wnt5a, Frizzled-5 and β-catenin during 4NQO-induced rat tongue carcinogenesis by immunohistochemistry. Male Wistar rats were distributed into three groups of 10 animals each and treated with 4NQO solution at 50 ppm through their drinking water for 4, 12, and 20 weeks. Ten animals were used as control group. No histopathological abnormalities were induced in the epithelium after 4 weeks of carcinogen exposure; however, an overexpression of Wnt5a was noticed when compared to control group ($p<0.05$). The Wnt1 showed significant differences ($p<0.05$) in pre-neoplastic lesions at 12 weeks following carcinogen exposure. In well-differentiated squamous cell carcinoma induced after 20 weeks of treatment with 4NQO, Wnt1 was expressed in the majority of the dysplasic cells and tumor cells. This was statistically significant ($p<0.05$). No significant differences ($p>0.05$) were found in expression of Frizzled-1, Frizzled-5 or β-catenin following oral carcinogenesis. Taken together, our results support the belief that expression of Wnt1 and Wnt5a is related to malignant transformation and conversion of oral mucosa.

Introduction

Oral cancer is a common neoplasm worldwide (Magrath and Litvak, 1993). It is desirable to examine the precise pathobiological mechanisms involved in oral tumorigenesis in order to identify reliable biomarkers for prevention of oral squamous cell carcinomas, especially during neoplastic conversion. A good experimental model for studying oral carcinogenesis is by water-soluble 4-nitroquinoline 1-oxide (4NQO) (Ribeiro and Salvadori, 2007). The medium-term assay is based on the multi-step process of carcinogenesis characterized by initiation, promotion, and tumor progression. Chronic administration of 4NQO in drinking water simulates rat tongue carcinogenesis similar to the human counterpart (Ohne et al., 1985; Nishimura, 1999; Niwa et al., 2001; Okazaki et al., 2002; Vered et al., 2003).

The Wingless-type (Wnt) protein family is a group of secreted glycoproteins, which play a pivotal role in regulation of proliferation and differentiation of epithelial cells involved in tumorigenesis of several tissues (Akiyama, 2000; Seidensticker and Behrens, 2000; Yamaguchi, 2001). The Wnt genes have been classified into functional groups with separate downstream signaling pathways. There is a canonical pathway and other non-canonical pathways. In the first, a multiprotein complex that includes adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and Axin ensure the degradation of β-catenin, thereby limiting the free intracytoplasmic pool of β-catenin in the absence of Wnt signaling. The presence of Wnt signal through the Frizzled (FZD) receptor and low density lipoprotein receptor-related protein 5 and 6 (LRP5/6) receptor complex inactivates GSK3 and causes its dissociation from Axin, preventing the phosphorylation of β-catenin (He et al., 2004). The intracytoplasmic pool of β-catenin thus increases and it translocates to the nucleus where it complexes with members of the LEF/TCF family of transcription factors to mediate transcriptional induction of target genes such as c-myc, cyclin D, VEGF and others (Logan and Nusse, 2004). In the second, Wnt signaling is transduced through Frizzled independent of LPR5/6. This pathway mediates cytoskeletal changes through activation of the small GTPases Rho and Rac in noncanonical or planar cell polarity (PCP) signaling (Habbas et al., 2005). The Wnt1 pathway signaling is thought to be mediated via interactions between β-catenin and members of the LEF-l/TCF family of transcription factors. It has been suggested as a possible role for the Wnt1 pathway in oral carcinogenesis (Lo Muzio, 2001). Also, this pathway appears to play an important role in basal cell carcinomas, as for example, areas of infiltration and/or of focal dedifferentiation of basal cell carcinoma (Lo Muzio, 2002). In this regard, the percentage

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