BIOMARCADORES DE SUSCETIBILIDADE EM CARCINOMAS DE VIAS AERODIGESTIVAS SUPERIORES

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LISTA DE ABREVIATURAS

CI	Confidence Interval
IC	Intervalo de Confiança
OR	*odds ratio*
ORs	*odds ratios*
UADT	*Upper Aerodigestive Tract*
VADS	Vias Aerodigestivas Superiores
ADH	*Alcohol dehydrogenase*
ADH	Álcool desidrogenase
ALDH	*Aldehyde dehydrogenase*
ALDH	Aldeído desidrogenase
CCND1	*Cyclyn D1*
CCND1	Ciclina D1
CYP	*Cytochromo P450*
CYP	Citocromo P450
GST	*Glutathione S-transferase*
GST	Glutationa S-transferase
NAT	*N-acetyltransferase*
NAT	N-acetiltransferase
NNN	*N'-nitrosonornicotine*
NNK	*4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone*
TSNA	*Tobacco-specific nitrosaminas*
TSNA	Nitrosaminas tabaco específicas

RESUMO

Nishimoto IN. **Biomarcadores de suscetibilidade em carcinomas de vias aerodigestivas superiores.** São Paulo; 2003 [Tese de Doutorado-Fundação Antonio Prudente]

O câncer das vias aerodigestivas superiores (VADS) está entre as formas mais comuns de neoplasias malignas, principalmente em países em desenvolvimento. Os hábitos de consumir tabaco e bebidas alcoólicas são os mais conhecidos e estabelecidos fatores de risco para o desenvolvimento dos carcinomas espinocelulares da boca, faringe e laringe. No entanto, somente poucos consumidores de bebidas alcoólicas ou de tabaco desenvolvem uma neoplasia de VADS. Por outro lado, outra pequena parte da população, não exposta aos carcinógenos do álcool e tabaco, podem desenvolver a doença. Estes fatos sugerem a existência de suscetibilidade genética individual. A carcinogênese envolve vários processos e recentes estudos têm identificado alguns genes associados com a suscetibilidade genética, perda do controle do ciclo celular, anormalidades no metabolismo de carcinógenos, reparo a danos no DNA e a tumorigênese das vias aerodigestivas superiores. Polimorfismos de genes envolvidos nessas vias patogênicas, como a ciclina D1 (*CCND1*) e a álcool desidrogenases (*ADH3*) apresentam-se como bons candidatos para a investigação de suscetibilidade genética. O risco de câncer de vias aerodigestivas superiores (VADS) pode ser atribuído ao consumo de álcool e às enzimas álcool desidrogenases (ADHs), que convertem o etanol em acetaldeído, um provável carcinógeno. Por outro lado, o envolvimento do gene da ciclina D1 (*CCND1*), localizada no cromossomo 11q13, apresenta importante papel na fase G1-S do ciclo celular. O polimorfismo *CCND1* G870A no códon 242 do exon 4 aumenta o *splicing* alternativo e desempenha uma importante função na formação das neoplasias. Para verificar a associação entre os polimorfismos dos genes *CCND1* e *ADH3* e o câncer de VADS, foram selecionados 147 casos (GC) (116 homens e 31 mulheres) e 135 indivíduos sem câncer (GSC) (91 homens e 44 mulheres). A localização dos tumores foi, 66 (44,9%) casos de boca; 43 (29,2%) de orofaringe; 7

(4,8%) de hipofaringe e 31 (21,1%) de laringe. O sistema *Wave™ System* foi usado para analisar o polimorfismo de cada genótipo. Dez por cento da amostras com perfil homozigoto e heterozigoto foram examinadas por sequenciamento direto. A análise estratificada, neste estudo mostrou riscos significativos para portadores do alelo A do polimorfismo da *CCND1*, OR = 7,5 para os que não consomem bebidas alcoólicas, e riscos não significativos para os fumantes OR = 5,4, ajustado por idade, gênero, consumo de tabaco e história familiar de câncer. Para o polimorfismo do *ADH3*, a análise estratificada e ajustada conferiu significativos riscos para o genótipo AA, OR=3.8 comparados ao combinado (GG+GA) para quantidades consumidas de etanol ao longo da vida inferior a 100 Kg e OR= 4,3 para os que consomem quantidades de tabaco inferior a 25 *packyears*. O genótipo AA do polimorfismo do gene do *ADH3* mostrou ser um possível fator de risco principalmente para os indivíduos que consomem baixas quantidades de álcool ou tabaco. Os resultados sugerem que a presença do alelo A do polimorfismo da *CCND1* confere riscos elevados especialmente para indivíduos sem história de consumo de bebidas alcoólicas.

SUMMARY

Nishimoto IN. **Biomarcadores de suscetibilidade em carcinomas de vias aerodigestivas superiores** [Biomarkers of susceptibility in upper aerodigestive system cancer]. São Paulo; 2003 [Tese de Doutorado-Fundação Antonio Prudente]

Cancers of the upper aerodigestive tract (UADT) are among the most common malignant neoplasms, particularly in developing countries. Although several investigations have shown that tobacco and alcohol are risk factors in the development of squamous cell carcinoma of the oral cavity, pharynx, or larynx, such cancers develop in only a small proportion of individuals who use tobacco and or alcohol, and some patients who have these cancers have had no apparent exposure to tobacco and alcohol. This suggests that host susceptibility may play an important role in SCC of the head and neck. The malignant process involves several steps, and recent studies have identified some genes associated with genetic susceptibility, metabolism of carcinogens, DNA repair, lost of cell cycle control and head and neck tumorigenesis. Polymorphisms in genes involved in these pathways, like cyclin D1 (*CCND1*) or alcohol dehydrogenases (*ADH3*) are candidate genes to be investigated for genetic susceptibility. The risks for development of upper aerodigestive tract (UADT) cancers may be attributable for alcohol consumption and alcohol dehydrogenase enzymes (ADHs), which convert ethanol to acetaldehyde. Otherwise, the involvement of cyclin D1 (*CCND1*), located within chromosome 11q13, plays a critical role in phase G1-S in the cell cycle control. The *CCND1* 870A/G polymorphism at codon 242 in exon 4 increases the alternate splicing, playing an important role in the formation of malignancies. To investigate the relationship between *CCND1* and *ADH3* polymorphisms on susceptibility for UADT cancers, 147 cases (116 male and 31 female) and 135 non-cancer cases (91 male and 44 female) were selected. The tumor sites cases were: oral cavity, 66 (44.9%); oropharynx, 43 (29.2%); hypopharynx, 7 (4.8%) and larynx, 31 (21.1%). The Wave™ System was used to polymorphisms analysis for each genotype. Ten percent of samples with

heterozygous and homozygous profiles were examined by direct sequencing to confirm the presence of the polymorphism. The stratified analysis of this study showed a significant higher odds ratio for *CCND1* polymorphism A allele OR=7.5 in non-drinkers and non-significant OR = 5.4 for smokers, adjusted for sex, age, tobacco use and first-degree family history of cancer. For *ADH3* polymorphism, the stratified and adjusted analysis showed significant higher odds ratio to AA genotypes OR=3.8 compared to GG+GA combined genotype for less than 100 kg ethanol lifetime consumers and OR=4.3 for less than 25 packyears tobacco smokers. These results suggest that *CCND1* combined A allele may be a risk factor especially in individuals without a history of drinking and that *ADH3* genotype AA may be a risk factor for subjects that are consumers of small quantities of ethanol or of tobacco smoking.

ÍNDICE

INTRODUÇÃO

1 INTRODUÇÃO

Baseado em recentes informações mundiais sobre a incidência e mortalidade devido às neoplasias malignas, estima-se a ocorrência de mais de 10 milhões de casos novos, com prevalência de 22 milhões de pessoas vivendo com câncer e de 6,2 milhões de óbitos no ano de 2000 (PARKIN et al. 2001). Desse total, estima-se que ocorram 267.000 casos novos, 128.000 mortes e prevalência de 707.000 casos de câncer da cavidade oral (PARKIN et al. 2001). A incidência do câncer bucal é extremamente variável e apresenta-se como um problema de saúde pública em muitas partes do mundo, sendo altamente prevalente na Índia e entre homens de algumas regiões da França (PARKIN et al. 1993). O risco de mortalidade tende a aumentar entre a população masculina em vários países (MACFARLANE et al. 1994).

O câncer de laringe representa 1,6% do total mundial, ou 161.000 casos novos dos quais 142.000 em homens; 89.000 mortes e 458.000 pessoas convivendo com a doença em 2000 (PARKIN et al. 2001). O Sul da Europa, Norte da África, Ásia Ocidental e América do Sul Temperada foram registrados como áreas de alto risco para o câncer de laringe (PARKIN et al. 2001). A elevada incidência esperada para a América do Sul Tropical decorre das altas taxas apresentadas nas regiões Sul e Sudeste do Brasil (PARKIN et al. 1993).

Os carcinomas espinocelulares das vias aerodigestivas superiores (VADS) representam menos de 5% das neoplasias nos Estados Unidos, entretanto, em países em desenvolvimento os cânceres de VADS estão entre as formas mais comuns da doença. Estima-se que nos Estados Unidos ocorram anualmente cerca de 41.000

casos novos de câncer de cabeça e pescoço (www.seer.cancer.gov) e no mundo aproximadamente 500.000 (PARKIN et al. 2001).

Segundo informações do MINISTÉRIO DA SAÚDE (2003), o câncer da cavidade oral figura como a sétima forma de neoplasia mais freqüente no Brasil em 2003, com 10.635 casos novos (3,2% de todos os casos, excluindo pele não melanoma), sendo 7.750 (8,9 por 100.000) para homens e 2.885 (3,2 por 100.000) mulheres. No Sudeste do Brasil espera-se a ocorrência de 5.190 casos novos, ocupando a quinta posição dentre as neoplasias dessa região entre os homens (MINISTÉRIO DA SAÚDE 2003). Conforme dados do Registro de Câncer de São Paulo, as taxas de incidência de câncer de boca, faringe e laringe no Município de São Paulo em 1969 foi de 45,3/100.000 habitantes, 48,0/100.000 em 1973, 56,3/100.000 em 1978 (MIRRA e FRANCO 1985), 55,4/100.000 em 1983, 52,1/100.000 em 1988 e 56,6/100.000 em 1993 (MIRRA 1999), 53,0/100.000 em 1997 e 45,7/100.000 em 1998 (MIRRA et al. 2001).

Para o câncer oral e orofaríngeo, a incidência estimada foi de 25,2 por 100.000 homens em São Paulo para 1993 (MIRRA 1999). As taxas de incidência para a neoplasia de VADS estão aumentando em São Paulo e em muitas áreas do mundo, provavelmente devido à contínua alta na prevalência do consumo de tabaco e bebidas alcoólicas (COLEMAN et al. 1993). É de se ressaltar que o aumento, tanto na incidência quanto na mortalidade, vem sendo notado em populações onde o tabaco não pode ser claramente identificado como principal agente causador, especificamente em adultos jovens com câncer oral (SHEMEN et al. 1984; DEPUE 1986, SCHANTZ et al. 1988, DAVIS e SEVERSON 1987, FRANCESCHI et al. 1994).

A maior parte dos tumores de cabeça e pescoço ocorre nas vias aerodigestivas superiores (principalmente boca, faringe e laringe), e são caracterizados por diferentes freqüências relativas de incidência e mortalidade em várias partes do mundo (VOKES et al. 1993). São geralmente estudados agrupadamente em epidemiologia, devido a:

- complexidade anatômica da região, que dificulta a identificação clara da origem de alguns tumores, principalmente quando o diagnóstico é feito em estádio avançado, sendo semelhantes em sua apresentação clínica, histologia e tratamento (SANGHVI et al. 1989; VOKES et al. 1993; TUPCHONG e ENGIN 1999).
- dois fatores de risco em comum que têm sido consistentemente identificados: o tabaco e o álcool (FRANCO 1987);

A devida magnitude de associação para os prováveis fatores de risco de neoplasias das vias aerodigestivas superiores é obtida através de dois tipos de investigações epidemiológicas: estudos de coortes (Risco Relativo) e estudos de caso-controle (Razão de Chances ou *Odds Ratio*) (BRESLOW e DAY 1980). Na primeira estratégia de estudo, um grupo de indivíduos com diferentes níveis de exposição aos fatores em estudo são acompanhados até o aparecimento da neoplasia, óbitos e/ou término do estudo, muitos anos após seu início. A razão entre as taxas de ocorrência de câncer para indivíduos expostos e não expostos a um determinado fator mede a magnitude das associações entre os fatores de risco e a doença. Esta medida é conhecida como risco relativo ou razão entre os riscos. Nos estudos caso-controle utilizam-se informações obtidas retrospectivamente. Os casos incidentes de uma doença são avaliados ao mesmo tempo que indivíduos controles selecionados entre a população hospitalar (controles hospitalares) ou na comunidade (controles

populacionais). A razão do risco entre os indivíduos expostos e não expostos é denominada de "*Odds Ratio*" (OR), palavra inglesa que é comumente conhecida no nosso meio como "Razão de Chances" ou também como "Razão dos produtos cruzados" (SOARES e BARTMANN 1985). Muitos fatores de riscos para cânceres de localizações anatômicas de vias respiratórias e de VADS resultam de estudos caso-controle com variadas formas de seleção de seus pacientes-controle.

Fumantes em potencial têm o risco aumentado de 3 a 20 vezes para os cânceres de cabeça e pescoço, dependendo do tipo do tabaco. Para pessoas que consomem elevadas quantidades de álcool têm-se estimado um aumento no risco que varia de 3 a 15 vezes, quando comparado àqueles que não bebem, dependendo da dose diária e localização do tumor (DOLL e PETO 1981; BURCH et al. 1981; DECKER e GOLDSTEIN 1982; DE STEFANI et al. 1988; FRANCO et al. 1989; BARRA et al. 1991; MUSCAT e WYNDER 1992; MUSCAT et al. 1996; SCHLECHT et al. 1999; KJÆRHEIM et al. 1998). Para o câncer de laringe, destacam-se outros fatores de risco como a exposição em residências equipadas com fogão a lenha, atividade ocupacional com madeira, histórico familiar de câncer (FOULKES et al. 1995) e alto consumo de chimarrão (WYNDER et al. 1956; VICTORA et al. 1987; DE STEFANI et al. 1987; SPITZ e NEWELL 1987; CATTARUZZA et al. 1996; PINTOS et al. 1998). Para o câncer oral destacam-se também a higiene, os cuidados de saúde bucal e dentição precária (GRAHAM et al. 1977; FRANCO et al. 1989; MARSHALL et al. 1992; VELLY et al. 1998).

Para o câncer de boca, elevadas estimativas de incidência em 1990 foram observadas na Melanésia e Austrália, que pode ser devido à prevalência do consumo de *betel-quid* no Centro Sul da Ásia e Melanésia, e a radiação solar e câncer de lábio

na Austrália, hábito de fumar tabaco e consumo de bebidas alcoólicas, que são as maiores causas, em países em desenvolvimento (PARKIN et al. 1999).

Alguns fatores de proteção têm sido associados aos cânceres de cabeça e pescoço, tais como o consumo de frutas cítricas e vegetais, principalmente aqueles ricos em caroteno (GRAHAM et al. 1977; CANN et al. 1985; SPITZ e NEWELL 1987; FRANCO et al. 1989; RAO et al. 1994; MARSHALL e BOYLE 1996; DE STEFANI et al. 1999a e b; NISHIMOTO et al. 2002).

Muitos estudos epidemiológicos demonstram que o meio ambiente e o estilo de vida são potenciais fatores de risco na etiologia do câncer. A relativa importância desses fatores e os mecanismos genéticos moleculares variam de indivíduo para indivíduo (WILD et al. 2002). Muitas neoplasias malignas resultam de fatores genéticos e ambientais, onde avaliações cuidadosas são necessárias para a compreensão dos mecanismos moleculares da carcinogênese humana, visando à prevenção primária e melhora no prognóstico do paciente (WILD et al. 2002).

Fatores Genéticos e Moleculares

A variabilidade inter individual de suscetibilidade pode ser explicada por alterações genéticas observadas no genoma humano. Diversos grupos têm se concentrado nas alterações, ou polimorfismos genéticos, que ocorrem nos genes codificadores para as enzimas metabolizadoras de carcinógenos (HARRIS 1987; BARTSCH et al. 2000).

Está bem estabelecido que o hábito de fumar tabaco é um dos principais fatores de risco em câncer de vias aerodigestivas superiores (VADS) e muitos

estudos têm demonstrado a influência das classes de carcinógenos dos hidrocarbonetos aromáticos policíclicos PAH (*Polycyclic Aromatic Hydrocarbon*), tais como CYPs e/ou GSTs, TSNA (*tobacco-specific nitrosaminas)* com NNK e/ou NNN e aminas aromáticas nos cânceres associados ao consumo de tabaco (BARTSCH et al. 2000). Em geral, a maioria dos agentes químicos carcinogênicos não produz os seus efeitos biológicos sem que ocorra a sua ativação por enzimas metabolizadoras. Desta forma, é provável que algumas dessas enzimas metabolizadoras de carcinógenos, que são encontradas no tabaco e nas bebidas alcoólicas, tenham um papel importante no desenvolvimento do carcinoma espinocelular de VADS. Exemplos destas enzimas incluem: CYPs, GSTs, NATs, ADHs e outros (HARRIS 1987; HAYASHI et al. 1991; KATO et al. 1994; HAYES and PULFORD 1995; JANKHE et al. 1996; HUNG et al. 1997; JOURENKOVA et al. 1998; JOURENKOVA-MIRONOVA et al. 1999a e b; HEIN et al. 2000; OLSHAN et al. 2000).

Conforme VINEIS et al. (1999), a introdução de estudos de polimorfismos na epidemiologia do câncer é "*particularmente importante por pelo menos três razões: a) identificação de uma sub-população de indivíduos que são mais suscetíveis ao câncer quimicamente-induzido e que aumentaria o poder de estudos epidemiológicos; b) a suspeita do papel de um agente etiológico é reforçada pelo conhecimento da enzima envolvida no seu metabolismo; c) os polimorfismos podem ser particularmente significativos em relação ao baixo nível de exposição, influenciando o processo de avaliação do risco e da determinação dos limites de "tolerância da exposição, que levaria em consideração a suscetibilidade individual*".

Embora o consumo de bebidas alcoólicas seja estabelecido como um potencial fator de risco para o desenvolvimento das neoplasias das vias aerodigestivas superiores, o seu mecanismo de ação e seu papel no câncer de VADS ainda não está completamente elucidado (LAZARUS e PARK 2000). Alguns estudos sugerem que polimorfismos dos genes que codificam enzimas tais como álcool desidrogenase e aldeído desidrogenase estejam envolvidos na patogênese dos cânceres de vias aerodigestivas superiores (boca, esôfago e laringe) (COUTELLE et al. 1997; LAZARUS e PARK 2000).

POLIMORFISMO do gene do *ADH3*

O mecanismo pelo qual o consumo de bebidas alcoólicas ou do etanol leva a carcinogênese ainda não está bem estabelecido (LAZARUS e PARK 2000). A hipótese de que polimorfismos no gene do álcool desidrogenase (*ADH*) influenciem na suscetibilidade em cânceres álcool-associados têm sido destacadas em estudos recentes (HARTY et al. 1997; OLSHAN et al. 2001; ZAVRAS et al. 2002). A classe I da família álcool desidrogenase (*ADH*), codificada pelos genes *ADH1, ADH2* e *ADH3* oxidam o etanol e outros álcoois alifáticos (FERGUSON e GOLDBERG 1997). Existem pelo menos sete tipos de álcool desidrogenase presentes nos humanos, entre esses a álcool desidrogenase tipo 3 (ADH3), que é uma enzima polimórfica que metaboliza o etanol em acetaldeído (MORRIS et al. 1989).

Alguns estudos avaliando o polimorfismo de *ADH3* como sendo um fator de risco para câncer de cabeça e pescoço têm sido realizados e seus resultados ainda mostram-se controversos (HARTY et al. 1997; WIGHT e OGDDEN 1998; BOUCHARDY et al. 2000; OLSHAN et al. 2001; SCHWARTZ et al. 2001).

O etanol é um dos principais componentes das bebidas alcoólicas, o qual é rapidamente absorvido através da mucosa gástrica e duodenal sendo metabolizado principalmente no fígado. O metabolismo hepático do álcool ocorre em 3 etapas (WIGHT e OGDEN 1998):

1. Oxidação do etanol para acetaldeído, principalmente da via álcool desidrogenase (ADH); (fase I, onde ocorre a presença ou ativação de enzimas oxidativas)

$$CH_3CH_2OH + NAD^+ \xleftrightarrow{ADH} CH_3CHO + NADH + H^+$$

2. Conversão do acetaldeído para acetato, que é catalisado através da enzima aldeído desidrogenase (ALDH); (fase II, onde ocorre a detoxificação que geralmente inativa os genotóxicos)

$$CH_3CHO + NAD^+ \xrightarrow{ALDH} CH_3CHOOH + NADH + H^+$$

3. O acetato é oxidado para produzir dióxido de carbono, ácidos saturados e água.

$$CH_3COOH \longrightarrow CH_3C(=O)\,ScoA$$

Em resumo, o possível mecanismo através do qual o consumo de álcool pode influenciar no desenvolvimento do câncer de cabeça e pescoço, é por meio da conversão de etanol para acetaldeído, que é um carcinógeno no trato respiratório animal (FERGUSON e GOLDBERG 1997).

A figura 1 ilustra um esquema simplificado e adaptado de WIGHT e OGDEN (1998), da conversão do álcool para o elemento carcinógeno, acetaldeído.

Figura 1: esquema simplificado da conversão do álcool para acetato

Além do etanol, nas bebidas alcoólicas podem ser encontrados as nitrosaminas e os contaminantes de uretano (WIGHT e OGDEN 1998). Algumas enzimas metabolizadoras de xenobiótico, encontradas também na mucosa das vias aerodigestivas superiores, podem ser carcinógenas e possivelmente atuam na formação de adutos e levariam a mutações do *p53* (FERGUSON e GOLDBERG 1997). Essas enzimas metabolizadoras de xenobiótico podem ativar as ADHs pela oxidação do etanol em acetaldeído, que é citotóxico e produz radicais livres e bases de DNA hidroxilados (SCULLY et al. 2000a).

Embora, somente uma pequena minoria de 10 a 20% de consumidores de bebidas alcoólicas venha a desenvolver uma neoplasia de VADS (WYNDER e BROSS 1957) e, uma outra pequena parte não exposta ao álcool isoladamente ou associada ao tabaco pode desenvolver a doença, sugerindo a existência de

suscetibilidade genética individual (COPPER et al. 1995; SCULLY et al. 2000a). A figura 2 representa um resumo da via carcinogênica no metabolismo do álcool.

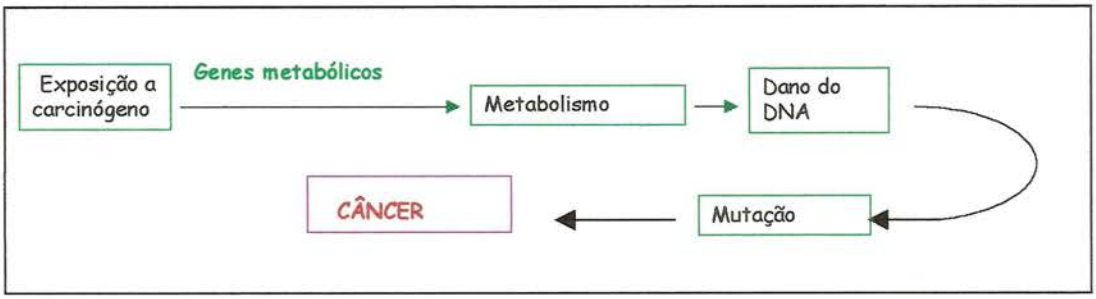


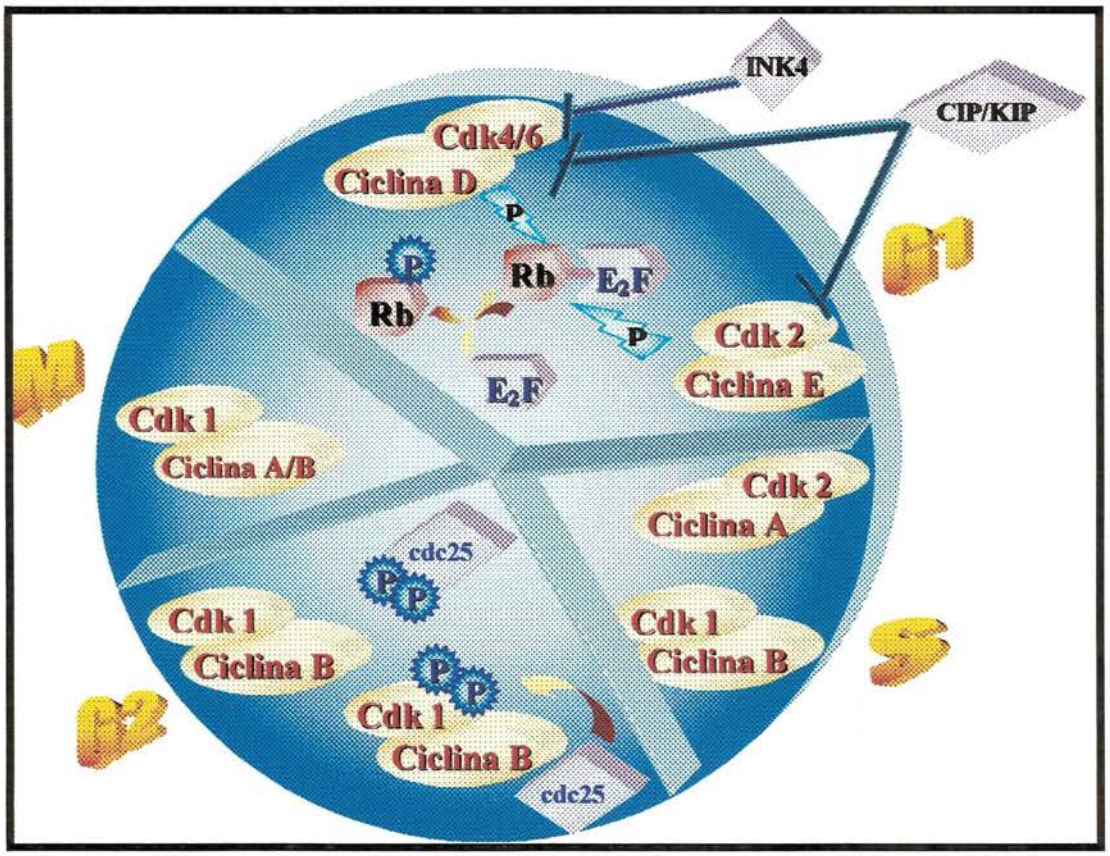
Figura 2 - Um esquema ilustrativo, adaptado de GARTE et. al. (1997), mostra um possível processo da carcinogênese, através do metabolismo do etanol.

POLIMORFISMO do gene da *CICLINA D1*

A carcinogênese de cabeça e pescoço pode ocorrer devido à anormalidades não somente no metabolismo de carcinógenos, mas também devido à alterações em genes de reparo a danos no DNA, genes envolvidos no controle do ciclo celular e apoptose. Polimorfismos de genes envolvidos nestes mecanismos e responsáveis pela manutenção da integridade celular, tais como o gene da ciclina D1 (*CCND1*), apresentam-se como bons candidatos para a investigação de suscetibilidade genética ao desenvolvimento de câncer (CALLENDER et al. 1994; XU et al. 1998; NAKASHIMA e CLAYMAN 2000).

A ciclina D1 (CCND1) é uma proteína reguladora importante no processo de transição da fase G1/S do ciclo celular, atuando preponderantemente na fase G1

associada as quinases dependentes de ciclinas CDK4 e CDK6 (JIANG et al. 1993; SHERR 1994). As CDKs são ativadas pelas ciclinas e inativadas pela família dos inibidores de CDKs, tais como as proteínas $p21^{CIP1}$, $p27^{kIP1}$ e $p57^{kIP2}$ (HUNTER e PINES 1994; SHERR 1996). A figura 3 ilustra um esquema simplificado da fase G1/S do ciclo celular e algumas proteínas envolvidas na via carcinogênica.



Fonte: Figura autorizada e reproduzida da dissertação de mestrado (MANGONE 2001).

Figura 3 - Esquema simplificado das fases do ciclo celular.

Como uma das principais ciclinas envolvidas nessa transição do ciclo celular, a CCND1 anormal pode atuar como um oncogene. A expressão aumentada desta proteína é freqüentemente observada em tumores humanos (CALLENDER et al. 1994; JARES et al. 1994). A sua ativação, devido à amplificação ou rearranjo cromossômico e conseqüente expressão aumentada, tem sido verificada em vários tumores, tais como carcinomas de mama, cabeça e pescoço, pulmão e colo-retal (JARES et al. 1994; SHAPIRO et al. 1995; GILLETT et al. 1996; MAEDA et al. 1998). O aumento da expressão da CCND1 pode levar a passagem celular prematura através da transição G1/S, resultando na propagação dos danos ao DNA, acúmulos de erros genéticos e a vantagem seletiva no crescimento para as células alteradas (HINDS et al. 1994). O complexo ciclina-CDK fosforila o gene supressor de tumor *RB*, inativando-o e acarretando a liberação do fator de transcrição E2F (*family of heterodimeric transcriptional regulators*). Desta forma, a transcrição pode ocorrer dando continuidade ao processo celular (HUNTER e PINES 1994; WEINBERG 1995; SHERR 1996). Considerando que as ciclinas constituem os componentes regulatórios do complexo ciclina-CDK, a alteração na expressão dessas proteínas pode ser elemento chave desse processo proliferativo desordenado (HUNTER e PINES 1994; WEINBERG 1995; SHERR 1996).

O gene *CCND1* está localizado no cromossomo 11q13. O polimorfismo deste gene, identificado como G870A cria uma forma alternativa de *splicing* e codifica para uma proteína com uma alteração no códon 242 do éxon 4, que não contem a seqüência envolvida na degradação e conseqüentemente tem uma vida média mais longa (BETTICHER et al. 1995).

A amplificação e a alteração da ciclina D1 têm sido relatadas como fatores prognósticos para câncer de cabeça e pescoço (MULLER et al. 1997; MATTHIAS et al. 1998; HOLLEY et al. 2001) e pulmão (JARES et al. 1994; SHAPIRO et al. 1995).

Embora, alguns recentes resultados vêm sendo descritos relacionando a presença do polimorfismo do gene *CCND1* como fator de risco no desenvolvimento do câncer de bexiga (WANG et al., 2002), colo-retal (KONG et al., 2001) e das VADS (ZHENG et al., 2001), poucos estudos epidemiológicos têm sido realizados com o objetivo de se verificar o polimorfismo do gene da ciclina D1 como possível fator de risco para o desenvolvimento do câncer.

OBJETIVOS

2 OBJETIVOS

Este estudo tem por objetivo principal investigar os polimorfismos *ADH3* e *CCND1* como possíveis fatores de risco para o desenvolvimento do câncer de vias aerodigestivas superiores, por meio da comparação entre um grupo de pacientes com diagnóstico confirmado de neoplasia maligna com um outro grupo de pacientes hospitalizados ou não, mas sem diagnóstico de neoplasias.

Pretende-se quantificar através da razão de chances ou *odds ratio* (OR), o papel da suscetibilidade genética mediada através dos polimorfismos genéticos:

1. *ADH3* (Ile349Val) e suas associações com relação ao consumo de bebidas alcoólicas e tabaco, como fator de risco para câncer de VADS, ajustando para os potenciais fatores de confusão;
2. verificar o genótipo *CCND1* G870A com relação ao consumo de bebidas alcoólicas e tabaco, como fator de risco para câncer de VADS, ajustando para os potenciais fatores de confusão.

3 ARTIGO 1

"Alcohol dehydrogenase 3 genotype as a risk factor for upper aerodigestive tract cancers"

O resumo deste trabalho foi apresentado no "Annual Meeting of the American Head and Neck Society"; Maio, 2003; Nashville,Tenn.

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Original Article

Alcohol Dehydrogenase 3 Genotype as a Risk Factor for Upper Aerodigestive Tract Cancers

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Running title: *ADH3* polymorphism for HNSCC

Key words: *ADH3* polymorphism, risk factor, cancer, upper aerodigestive system

SUMMARY

Objective: *Alcohol dehydrogenase 3* (*ADH3*) polymorphism at position Ile349Val as indicator of risk factor for upper aerodigestive tract (UADT) cancer was assessed to verify its association with UADT cancer in nonalcoholic or nonsmoking individuals. **Design:** Cross-sectional study. **Setting:** Primary care or referral center. **Patients:** The study group consisted of 141 consecutive patients with newly diagnosed squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx, admitted for surgical treatment. The comparison group consisted of 94 inpatients without cancer from the A. C. Camargo Hospital or other São Paulo hospital and 40 healthy individuals. **Intervention:** All participants were interviewed and data collected using a structured questionnaire. After written informed consent was obtained, 20 mL of blood were collected in heparinized tubes. **Main Outcome Measures:** Odds ratio for ADH3 genotypes using logistic regression models. **Results:** After adjustment for sex, age, tobacco use, and first-degree family history of cancer, a significantly higher odds ratio for UADTcancer was observed among individuals with AA genotype and low cumulative alcohol consumption (≤100 kg of ethanol) (OR = 3.8 [95% confidence interval, 1.5-9.7]). A 4-fold increase in odds ratio for UADT cancer among individuals with AA genotype and low tobacco consumption (≤25 pack-years) was also found in the adjusted model. **Conclusions:** These results suggest that genotype AA may be a risk factor for UADT cancer, especially in individuals with low alcohol or tobacco consumption. However, further epidemiological case-control or cohort studies, preferably prospective, are needed to establish the exact role of *ADH3* polymorphism and its association with the development of UADT cancers.

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Incidence rates of head and neck cancer in southern Brazil are among the highest in the world, in both men and women (FRANCESCHI et al. 2000) and the rates of squamous cell carcinoma (SCC) of oral, pharyngeal, and laryngeal sites are rising among men in many countries (COLEMAN et al. 1993; FRANCESCHI et al. 2000; MOORE et al. 2000; WUNSCH-FILHO 2002).

Upper aerodigestive tract (UADT) cancers are a heterogeneous group of neoplasms with similar etiology (VOKES et al. 1993). Although several epidemiologic investigations have shown tobacco and alcohol to be risk factors in the development of SCC of the head and neck, such cancers develop in only a small proportion of individuals who use tobacco and alcohol, and some patients who have these cancers have had no apparent exposure to tobacco and alcohol. This suggests that host susceptibility may play an important role in SCC of the head and neck (COPPER et al. 1995; SCULLY et al. 2000a).

Several studies have shown an increased risk for UADT cancers among heavy drinkers or nonsmoking alcohol consumers compared with abstainers (SCHLECHT et al. 1999). Chronic alcohol consumption is correlated with carcinogenesis through a variety of mechanisms, and the basal and epithelial cells of UADT cancers may also be damaged by high ethanol concentrations on the exposed mucosae, leading to higher carcinogenic and procarcinogenic concentrations (SIMANOWSKI et al. 1995; SCULLY et al. 2000a).

The influence of alcohol consumption on the risk of cancer may be due to mucosal alcohol dehydrogenase (ADH) or to oropharyngeal bacteria that convert ethanol to acetaldehyde, a possible UADT carcinogen (SIMANOWSKI et al. 1995). There are polymorphic variants of *ADH* and *ADH3*, which is known to produce

enzymes with distinct kinetic properties, can cause variation in the capacity of individuals to metabolize alcohol (FERGUSON e GOLDBERG 1997). Studies have shown controversial results regarding *ADH3* polymorphism as a possible risk factor for SCC of the head and neck (COUTELLE et al. 1997; BOUCHARDY et al. 2000; STURGIS et al. 2001; OLSHAN et al. 2001).

Given that tobacco and alcohol consumption are the most important risk factors for UADT cancers, *ADH3* polymorphism at position Ile349Val was assessed as a risk factor for UADT cancers in nonalcoholic, alcoholic, nonsmoking, and smoking study subjects.

METHODS

From 1995 to 1998, 141 consecutive patients newly diagnosed with SCC of the oral cavity, oropharynx, hypopharynx, or larynx who were admitted for surgical treatment at the Department of Head and Neck Surgery and Otorhinolaryngology of the Hospital do Câncer A. C. Camargo, São Paulo, Brazil, were enrolled in this study. The sites of these unpreviously treated tumors were the oral cavity (n = 63 [44.7%]); oropharynx (n = 41 [29.1%]); hypopharynx (n = 8 [5.7%]), and larynx (n = 29 [20.6%]).

From 1995 to 2001, 2 unmatched groups of cancer-free individuals were selected as comparison groups: 40 healthy volunteers, and 94 inpatients from the A. C. Camargo or other São Paulo hospital who had diseases other than cancer (3 patients had infectious diseases; 22 had benign neoplasms; 4 had endocrine diseases;

20 had diseases of the cardiovascular system; 1 had asthma; 31 had diseases of the digestive system [there was only 1 case of hepatic cirrhosis]; 4 had diseases of the genitourinary tract; 1 had osteomuscular disease; 2 had congenital disorders; 2 had ill-defined diagnostic conditions, and 4 had received traumas).

A trained nurse interviewed all participants using a structured questionnaire, to obtain detailed information on socioeconomic and demographic characteristics and on lifetime tobacco and alcoholic beverage consumption. The study protocol was approved by the Institutional Ethic Committee. After participants had given written informed consent, 20 mL of blood were collected from them in heparinized tubes.

A leukocyte pellet was extracted from each blood sample with the Wizard Genomic DNA Purification Kit (Promega, 4 Madison, Wis) to obtain genomic DNA. Polymorphism analysis was performed after amplification by polymerase chain reaction (PCR). The PCR amplification was performed using the primers, 5'-CTT-TAA-GAG-TAA-AGA-ATC-TGT-CC-3' (sense) and 5'- ACC-TGC-TTC-GCT-CTG-GAA-AGA-G-3' (antisense) for genotyping *ADH3* polymorphism at codon 349, which generated 140-bp fragments. These fragments were amplified in 25 μL of reaction mixture containing about 100 ng of genomic DNA, 6.25 pmoL of each primer, 1 × PCR buffer (20mM Tris hydrochloric acid (pH 9.0), 50mM potassium chloride, 3mM magnesium chloride, 0.2mM of each of the deoxynucleotides adenosine triphosphate, thymidine triphosphate, guanine triphosphate, cytosine triphosphate, and 1 U of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystem, Foster City, CA). The reactions were performed by incubating the reaction mixture at 94°C for 10 minutes, followed by 35 cycles at 94°C for 45 seconds and at 65°C for 1 minute, then at 72°C for 1 minute, with a final elongation step at 72°C for 7 minutes.

Polymorphism analysis was performed with the Wave™ system (Transgenomic Inc, Omaha, Neb), after the PCR products were denatured at 94°C for 5 minutes and cooled to 65°C, with a temperature change of 1°C per minute. The samples were then kept at 4°C until 5 µL were injected in the column, and the PCR products were separated over 8 minutes (flow rate, 0.9 mL/minute) through a linear acetonitrile gradient automatically determined by the size and G-C content of the fragment as described by UNDERHILL et al. (1997). The optimal melting temperature for PCR fragments analysis was 63°C.

Every sample in which only 1 peak was identified was further analyzed by preparing equimolar mixtures with PCR products from known wild-type samples to potentially generate heteroduplex DNA. After denaturation, reannealing, and re-injection, the appearance of heterozygous elution profiles after addition of the wild-type sample suggested the presence of a homozygous mutant genotype.

Only 10% of samples with heterozygous and homozygous profiles were examined by direct sequencing to confirm the presence of the polymorphism because it is known that the same polymorphism gives identical chromatographic tracing among individuals, and common polymorphisms can be identified by pattern recognition alone (YOUNG et al. 2002).

The sequence analysis was performed using the Dynamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Piscataway, NJ).

Genotype analysis was performed for 148 subjects with cancer and 137 subjects without cancer, but the ADH3 genotype could not be determined for 7 subjects with cancer and 3 without.

For univariate data analysis, the associations of categorical data were determined using the frequency χ_2 tests and 2 × 2 tables; when the expected frequency was less than 5, the Fisher exact test was used. The Hardy-Weinberg equilibrium analysis ($p_2 + 2pq + q_2$;, with $q = 1-p$) was performed to estimate the expected genotype distribution (AA, GA, GG), which was compared with observed genotype frequencies using a χ_2 test. Unconditional logistic regression with 95% confidence interval (CI) was performed to obtain the estimated odds ratio (OR) between genotype and UADT cancer (BRESLOW e DAY 1980). Owing to the small number of subjects with the homozygous G allele, those who had GG genotype were grouped with those who had an heterozygous allele (GA or GG). A multivariate logistic regression model was used, controlling for the confounding effects of age, sex, lifetime tobacco and alcohol consumption, and cancer history in the first-degree family (no/yes answer).

Lifetime consumption of alcohol was collected for 4 types of alcoholic beverages: beer, wine, hard liquor, and cachaça, a spirit distilled from sugar cane. Cumulative alcohol exposure was expressed in kilograms of ethanol calculated from the consumption of individual beverages over the patients entire life (FRANCO et al. 1989). As an expression of cumulative exposure, the consumed amount of ethanol in kilograms was combined with drinking frequency and years of drinking into a synthetic index. A dose of ethanol consumed corresponded to 5% for beer, 10% for wine, 50% for hard liquor, or 50% for cachaça (FRANCO et al. 1989).

Cumulative exposure to tobacco smoking was expressed in pack-years, defined as the cumulative exposure equivalent to packs of cigarettes smoked per day multiplied by the number of years the patient smoked. For the computation of pack-

years of tobacco consumption we assumed the following equivalence: 1 pack equals 20 industrial cigarettes, 4 hand-rolled cigarettes of black tobacco, 4 cigars, or 5 pipefuls of pipe tobacco (FRANCO et al. 1989). Medians or quartiles were considered cut-offs for continuous variables. All statistical analyses were performed using the statistical software package Stata, release 7.0 (Stata Corp, College Station, Tex).

RESULTS

This investigation was based on 141 subjects (110 male and 31 female) with UADT cancer and 134 subjects (91 male and 43 female) without cancer. Those without cancer (median age, 58 years; range, 22-90 years) were likely to be younger than those with cancer (median age, 53 years; range, 17-90 years). The main characteristics of the participants, such as demographics, lifestyle variables, and ADH3 genotype distribution, are presented in Table 1.

The distribution of the *ADH3* genotypes: AA, GA, and GG was similar in the group with cancer (51.8%, 41.8%, and 6.4%, respectively) and in the group without cancer (48.5%,; 44.0%, and 7.5%) ($P = 0.84$). The genotype distribution in the group without cancer was consistent with the Hardy- Weinberg equilibrium ($P = 0.84$). Table 2 shows the distribution of genotypes and demographic and lifestyle variables. No statistically significant P value was obtained by χ_2 test regarding the effects of the study variables on genotypes GA and GG on the one hand, and AA on the other. Univariate and multivariate logistic regression analyses of genotypes provided

nonsignificant results, and there were no significant associations between genotype and cancer site (oral cavity, pharynx or larynx) (data not shown in tables).

In stratified analysis, the logistic regression model, adjusted for potential confounders (age, sex, family history of cancer, and tobacco and alcohol consumption), showed a high risk of UADT cancer for subjects with homozygous AA genotype and low cumulative alcohol consumption (≤100 Kg of ethanol) (OR, 3.8; 95% CI, 1.5-9.7). However, for subjects with a cumulative alcohol consumption greater than 100 Kg of ethanol, AA genotype was nonsignificant (OR, 0.5) (Table 3). Also, there was a significant 4-fold OR increase in subjects with AA genotype and low tobacco consumption (< 25 pack-years); and a negative OR was found for heavy smokers, but a nonsignificant OR of 0.4 (95% CI, 0.2-1.0) was found for heavy smokers with AA genotype (Table 3).

COMMENT

Alcohol dehydrogenase 3 polymorphism at Ile349 Val as a risk indicator for UADT cancers was assessed to verify its association with cancer in individuals with low levels of alcohol or tobacco consumption.

Analysis stratified by alcohol and tobacco consumption showed *ADH3* polymorphism as an added risk for individuals with the AA genotype and low tobacco smoking or alcohol intake, but nonstratified logistic regression analysis showed no significant added risk BOUCHARDY et al. (2000), verified the role of ADH3 genotype in patients with UADT cancer. The authors examined the adenine to

guanine change (Ile349Val amino acid substitution), but observed no association between genotype and UADT cancer; however, they showed strong interaction between *ADH3* and high alcohol consumption on the one hand and oral cavity/pharyngeal cancers on the other. In another recent study, OLSHAN et al. (2001) examined differences between the Ile (*ADH3*1*) and Val (*ADH3*2*) codons at amino acid residue 349 in head and neck cancer patients, but found no significant associations. Also, they found no joint effects or interaction between the ADH3 genotype and alcohol consumption. By contrast, the results of a study recently conducted by ZAVRAS et al. (2002) on the population of Athens, Greece, suggest an interaction of genotype and environment in oral cancer. Because of our small sample size, we could not verify interaction between genotype and phenotype in this study.

Our study proposed to verify the association between alcohol or tobacco consumption and *ADH3* polymorphism at position Ile349Val as an indicator of risk for UADT cancers. We observed that 37 (26%) subjects in our group with cancer and 96 (61%) subjects in our group without cancer reported a low consumption of alcoholic beverages, and a similar distribution was observed for low tobacco consumption. Head and neck cancer is rare in nonsmokers and alcohol abstainers, and only a few studies have included a sufficient number of patients with cancer (FIORETTI et al. 1999; TALAMINI et al. 1998). Chronic ethanol consumption has been associated with malnutrition and with inadequate diets deprived of known cancer-protective elements (SIMANOWSKI et al. 1995). Because only tobacco and alcohol consumption were evaluated in this study, we were not be able to control for fruit and vegetable consumption, which has been shown to be protective factors for UADT cancers (FRANCO et al. 1989; NISHIMOTO et al. 2002).

Approximately 70% of our participants without cancer were selected from the same hospital as those with cancer or from a neighborhood hospital, and 30% were healthy volunteers. However, the observed ADH3 genotype distribution and that expected from the Hardy-Weinberg equilibrium model suggested no selection bias ($P>.05$, χ_2 test). In spite of the small sample size, and the fact that groups were not matched by sex, age, and cancer family history, stratified analysis showed significant added risk of UADT cancers in individuals with ADH3 AA genotype.

Furthermore, our results showed a significant 4-fold risk increase for UADT cancers in individuals with ADH3 AA genotype and low consumption of alcohol or tobacco, but, contrary to our expectations, our results also showed heavy consumption of alcool and smoking tobacco to be protective; nonsignificant ORs, however, were obtained. The various xenobiotic-metabolizing enzymes found in the mucosae of the upper aerodigestive tract are involved in activating the metabolism of carcinogens or procarcinogens (SCULLY et al. 2000a). Phase I enzyme oxidative alcohol dehydrogenase is responsible for carcinogen activation, there are many isoforms of ADH, and other genes are also involved. Although there are phase II detoxification enzymes, which generally inactivate the genotoxic compounds, aldehyde dehydrogenase is involved in the carcinogen phase II pathway in ethanol metabolism (STURGIS e WEI 2002). The ADH3 gene (a class I ADH gene) plays a major role in ethanol oxidation (FERGUSON e GOLDBERG 1997), and the results of this study place genotype AA of *ADH3* polymorphism as a candidate gene for head and neck cancer risk in individuals with low alcohol or tobacco consumption.

Although increased risks were detected in this study, data could have been confounded by the differences between groups for adjusted variables. Study power

and risk significance may have been more convincing if the study design had been case-control. Therefore, the results need to be confirmed in further studies, large enough to provide statistical power, preferably matched (by age, sex, cancer family history) case-control or prospective epidemiological cohort study, to establish the exact role of *ADH3* polymorphism. Multiple gene interaction, genotype-environment interaction, and the systemic and topic effects of the presence or absence of tobacco and alcohol should be considered, and their association with the development of UADT cancers.

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Table 1 - Distribution of 141 cancer cases and 134 non-cancer cases according to demographic and lifestyle variables and *ADH3* genotypes.

Variables	Levels	Subjects Non-Cancer Freq. (%)	Cancer Freq. (%)	p-value*
Age (years)	≤ 55	79 (59.0)	56 (39.7)	0.001
	> 55	55 (41.0)	85 (60.3)	
Gender	Male	91 (67.9)	110 (78.0)	0.059
	Female	43 (32.1)	31 (22.0)	
Race	Caucasian	110 (82.1)	119 (84.4)	0.608
	Non-Caucasian	24 (17.9)	22 (15.6)	
1st.Degree Cancer	No	105 (78.4)	77 (54.6)	<0.001
Family history	Yes	29 (21.6)	64 (45.4)	
Tobacco	≤ 1	67 (50.0)	15 (10.6)	<0.001
(pack years)	1 – 25	26 (19.4)	31 (22.0)	
	26 - 50	23 (17.2)	49 (34.8)	
	>50	18 (13.4)	46 (32.6)	
Lifetime alcohol	≤ 1	68 (50.7)	21 (14.9)	<0.001
Consumption	1 – 100	28 (20.9)	16 (11.3)	
(Kg of ethanol)	>100	38 (28.4)	104 (73.8)	
ADH3 Genotype	AA	65 (48.5)	73 (51.8)	0.844
	GA	59 (44.0)	59 (41.8)	
	GG	10 (7.5)	9 (6.4)	

* p-value obtained from chi-square test

Table 2 - Distribution of *ADH3* genotypes according to demographic and lifestyle variables.

Variables	Levels	*ADH3* genotypes AA Freq. (%)	GA + GG Freq. (%)	p-value*
Age (years)	≤ 55	73 (52.9)	62 (45.3)	0.205
	> 55	65 (47.1)	75 (54.7)	
Gender	Male	99 (71.7)	102 (74.4)	0.612
	Female	39 (28.3)	35 (25.6)	
Race	Caucasian	110 (79.7)	119 (86.9)	0.112
	Non-Caucasian	28 (20.3)	18 (13.1)	
1st. Degree cancer	No	94 (68.1)	88 (64.2)	0.496
Family history	Yes	44 (31.9)	49 (35.8)	
Tobacco consumption	≤ 1	44 (31.9)	38 (27.7)	0.646
(Pack years)	1 – 25	31 (22.5)	26 (19.0)	
	26 – 50	34 (24.6)	38 (27.7)	
	>50	29 (21.0)	35 (25.6)	
Lifetime alcohol	≤ 1	45 (32.6)	44 (32.1)	0.996
consumption	1 – 100	22 (15.9)	22 (16.1)	
(kg of ethanol)	>100	71 (51.5)	71 (51.8)	
Tumor	Oral cavity	26 (35.6)	37 (54.4)	0.084
localization	Oropharynx	22 (30.1)	19 (27.9)	
	Hypopharynx	5 (6.9)	3 (4.4)	
	Larynx	20 (27.4)	9 (13.2)	

* p-value obtained from chi-square test

Table 3 - The Odds ratio OR (95% CI) of the UADT cancer according to *ADH3* genotypes and lifetime alcohol (Kg of ethanol) and tobacco consumption (pack years).

Stratified models	**Adjusted OR* (95% CI)**	p
Alcohol consumption ≤ 100g ethanol		
AA versus (GG + GA)	3.77 (1.5 – 9.7)	0.006
Tobacco > 25 pack year	5.53 (1.8 – 16.6)	0.002
Alcohol consumption > 100kg ethanol		
AA versus (GG + GA)	0.52 (0.2 – 1.2)	0.120
Tobacco > 25 pack year	1.30 (0.5 – 3.2)	0.569
Tobacco ≤ 25pack year		
AA versus (GG + GA)	4.27 (1.7 – 10.8)	0.002
Alcohol consumption > 100kg ethanol	14.3 (4.6 – 44.5)	<0.001
Tobacco > 25 pack year		
AA versus (GG + GA)	0.43 (0.2 – 1.0)	0.050
Alcohol consumption > 100kg ethanol	6.71 (2.2 – 20.9)	0.001

* adjusted by gender, first degree cancer family history and age (≤ 55, >55)

* Odds ratio obtained by logistic regression model

4 ARTIGO 2

" Cyclin D1 gene polymorphism as a risk factor for squamous cell carcinoma of the upper aerodigestive tract system in non-alcoholics",

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CYCLIN D1 GENE POLYMORPHISM AS A RISK FACTOR FOR SQUAMOUS CELL CARCINOMA OF THE UPPER AERODIGESTIVE SYSTEM IN NON-ALCOHOLICS.

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SUMMARY

Squamous cell carcinoma of the upper aerodigestive tract (UADT) is associated with environmental factors, especially tobacco and alcohol consumption. Genetic factors, including cyclin D1 (*CCND1*) polymorphism have been suggested to play an important role in tumorigenesis and progression of UADT cancer. To investigate the relationship between *CCND1* polymorphism on susceptibility for UADT cancers, 147 cancer and 135 non-cancer subjects were included in this study. *CCND1* genotype at codon 242(G870A) in exon 4 was undertaken using Denaturing High Performance Liquid Chromatography (DHPLC) and DNA sequencing. Significant odds ratio (OR) of the AA+GA genotypes [OR=7.5 (95% CI: 1.4-39.7)] was observed in non-drinkers but for non-smokers a non-significant [OR=5.4 (95% CI: 0.9-31.4)] was found in the adjusted model. These results suggest that allele A may be a risk factor for UADT cancer, especially in non-alcoholics. However, further epidemiological studies are needed to establish the exact role of *CCND1* polymorphism and the development of UADT cancers.

Key words: UADT, *CCND1* polymorphism, risk factor, cancer, upper aerodigestive system

INTRODUCTION

Carcinomas of the upper aero-digestive tract (UADT) are among the most common neoplasms, particularly in developing countries and affect approximately 500,000 new persons per year worldwide (PARKIN et al. 2001). Incidence rates of oral cancer in Southern Brazil are among the highest in the world in both for men and women (FRANCESCHI et al. 2000), and the rates of squamous cell carcinoma of oral cavity, pharyngeal and laryngeal sites are rising among men in many countries (FRANCESCHI et al. 2000; WUNSCH-FILHO 2002).

Squamous cell carcinomas (SCC) of the UADT are a heterogeneous group of neoplasms with similar etiology, strongly related to environmental factors, such as tobacco and alcohol consumption (FRANCO et al. 1989; WUNSCH-FILHO 2002). Although the considerable importance of the use of tobacco and alcohol as risk factors for SCC of the UADT, only a small proportion of the exposed individuals develop cancer, and some patients who have these cancers have no apparent exposure to tobacco and alcohol. This suggests that host susceptibility may play an important role in UADT carcinogenesis (COPPER et al. 1995; SHERR 1996; SCULLY et al. 2000a).

The malignant process involves several steps, and recent studies have identified some genes associated with genetic susceptibility, lost of cell cycle control and head and neck tumorigenesis (COPPER et al. 1995; SHERR 1996; MULLER et al. 1997; MATHIAS et al. 1999; SCULLY et al. 2000a e b; GLEICH e SALAMONE 2002).

The cell cycle is linked to tumorigenesis and the deregulation of G1-S phase progression of the cell cycle is a frequent target in malignant transformation (SHERR 1996, SAWA et al., 1998). Cyclin D1 gene (*CCND1*) is a key regulator of G1 phase of the cell cycle. The transition through G1 into S phase is regulated by cyclin dependent kinases (CDKs) CDK4 and CDK6, in protein complexes with cyclin D1. Inhibition of cyclin D1 function results in cell-cycle arrest, whereas unregulated expression of the protein accelerates the G1 phase (HUNTER e PINES 1994; SHERR 1996).

CCND1 gene encodes cyclin D1, which is a protein which catalyzes the phosphorylation of the tumor suppressor protein retinoblastoma (RB), leading to the dissociation of RB, which results in the release of a family of heterodimeric transcriptional regulators E2F from RB and reversing RB functional inactivation (HUNTER e PINES 1994; SHERR 1996). *CCND1* is frequently amplified and over-expressed in a variety of tumor types and induces proliferation (SHERR 1996; GLEICH e SALAMONE 2002). In head and neck squamous cell carcinomas (HNSCC), the *CCND1* has been shown amplified or over expressed in 12 to 68% of tumors using different techniques, such as fluorescence in situ hybridization (FISH), Southern blotting and immunohistochemistry (AKERVALL et al. 2002; GLEICH e SALAMONE 2002). Studies that showed a relationship between *CCND1* and outcome found that amplification or overexpression was associated with recurrence, nodal metastasis and death (DONNELLAN and CHETTY et al. 1998; GLEICH e SALAMONE 2002).

CCND1 has a G to A polymorphism at codon 242 (G870A) in exon 4, which increases the frequency of alternative splicing, leading to an altered protein that does

not contain the sequence involved in protein turnover and then has a longer half-life (BETTICHER et al. 1995; KONG et al. 2000). It has been suggested that DNA damage in cells from subjects with the A allele may bypass the G1/S checkpoint of the cell cycle control mechanism more easily than in cells not carrying the polymorphism (BETTICHER et al. 1995; KONG et al. 2000). Recent studies have shown that *CCND1* polymorphism is a possible risk factor to HNSCC (ZHENG et al. 2001); colorectal cancer at young age (KONG et al. 2001); familial and sporadic colorectal cancer (PORTER et al. 2002) and urinary bladder cancer (WANG et al. 2002).

Given that tobacco and alcohol consumption are the most important risk factors to UADT cancers, *CCND1* polymorphism was assessed as a risk factor for UADT cancers in non-alcoholics or non-smoking subjects.

PATIENTS

From 1995 to 2001, 147 patients newly diagnosed and histologically confirmed SCC of the oral cavity, oropharynx, hypopharynx and larynx, who were admitted for surgical treatment at the Head and Neck Surgery and Otorhinolaryngology Department, of the Hospital do Câncer A.C.Camargo, Brazil, were enrolled to this study. The sites of these previously untreated tumors were: oral cavity, 66 (44.9%); oropharynx, 43 (29.2%); hypopharynx, 7 (4.8%) and larynx, 31 (21.1%). According to the tumor staging system of the American Joint Committee on Cancer, 45 (30.6%) cases presented small tumors (stage T1 or T2), 53 (36.0%) had T3, 47 (32.0%) T4 and in 2 cases the stage T was unrecorded (Tx). There were

72 (49.0%) cases with negative lymph nodes, 74 (50.3%) with clinically metastatic lymph nodes, and in one case it was not possible to evaluate (Nx).

Two unmatched groups of cancer-free individuals were selected as comparison group: 41 subjects were healthy volunteers (blood donors or participants on physical checkups) and 94 inpatients from the Hospital do Câncer A.C.Camargo, a reference institution, or other neighborhood general hospitals in Sao Paulo who had diseases other than cancer (3 patients had infectious diseases; 22 benign neoplasms; 4 endocrine diseases; 22 diseases of the cardiovascular system; 1 asthma; 30 diseases of the digestive system [there was only one case of hepatic cirrhosis]; 4 diseases of the genitourinary tract; 2 congenital disorders; 2 ill-defined diagnostic conditions, and 4 traumas).

A trained nurse interviewed all participants using a structured questionnaire to obtain an accurately and detailed information on demographic characteristics, any first-degree cancer family history, and on lifetime habits of tobacco and alcoholic beverage consumption.

The institutional ethic committee approved the study protocol. After participants had given a written informed consent, 20 ml of blood were collected from them in heparinized tubes.

METHODS

From each blood sample a leukocyte cell pellet was obtained by centrifugation and used for DNA extraction with the Wizard® Genomic DNA

Purification Kit (Promega-Madison, Wis, USA), according to the manufacturer's instructions to obtain genomic DNA. *P*olymorphism analysis was performed after amplification by polymerase chain reaction (PCR).

The PCR amplification was performed using the primers, 5'-TAC-TAC-CGC-CTC-ACA-CGC-TTC-C-3' (sense) and 5'-TTG-GCA-CCA-GCC-TCG-GCA-TTT-C-3' (antisense), which generate a 138 bp fragment. These fragments were amplified in 25µl of reaction mixture containing about 50ng of genomic DNA, 6.25 pmol each primer, 1x PCR buffer (20mM Tris-HCl, pH 9.0, 50mM KCl), 1.5mM $MgCl_2$, 0.2mM each dATP, dTTP, dGTP and dCTP and 1U of *Taq polymerase* (AmpliTaq Gold®: Applied Biosystem, Foster City, CA, USA). The reactions were performed by incubating the reaction mixture at 94°C for 10 min, subjecting them to 35 cycles of 94°C for 45s and 65°C for 1 min and then at 72°C for 1 min, with a final elongation step at 72°C for 7 min.

Polymorphism analysis was performed with the Wave™ System (Transgenomic Inc, Omaha, Neb), after the PCR products were denatured at 94°C for 5 min and cooled to 65°C with a temperature change of 1°C/min. The samples were then kept at 4°C until 5 µl were injected to the column. PCR products were then separated (flow rate of 0.9 ml/min) over 8 min and through a linear acetonitrile gradient, determined automatically by the size and G-C content of the fragment as described by UNDERHILL et al. (1997). The optimal melting temperature for these PCR fragments analysis was 63°C.

Every sample in which only one peak was identified was further analyzed by preparing equimolar mixtures with PCR products from known wild-type samples to generate potential heteroduplexes species. After denaturation, reannealing and re-

injection, the appearance of heterozygous elution profiles after the addition of the wild-type sample suggests the presence of a homozygous mutant genotype.

Only ten percent of samples with heterozygous and homozygous profiles were examined by direct sequencing to confirm the presence of the polymorphism because it is known that the same polymorphism gives identical chromatographic tracing among individuals, and common polymorphisms can be identified by pattern recognition alone. The sequence analysis was performed using the Dynamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ).

The genotype analysis was performed for 148 cancer cases and 137 non-cancer subjects, but *CCND1* genotype could not be determined for one cancer case and two non-cancer individuals.

The associations between variables were determined by chi-square and Fisher exact test. The Hardy-Weinberg equilibrium analysis ($p^2+2pq+q^2$; where q=1-p) was performed to estimate the expected genotype distribution (GG, GA, AA), which was compared with observed genotype frequencies using a chi-squared test. Unconditional logistic regression with 95% confidence interval (95% CI) was performed to obtain the estimated odds ratio (OR) between genotype and UADT cancer (BRESLOW e DAY 1980).

Owing to the small number of subjects with the homozygous A allele, they were grouped with those who had a heterozygous allele (GA or AA). Multivariate logistic regression models were used controlling for confounding effects of age (as continuous variable), gender, tobacco and alcohol habits (never or ex/ever), and cancer history in the first-degree family (no/yes answer). The genotype GG (wild

type genotype) was considered as a reference category. All analysis was performed using the statistical software package STATA release 7.0.

RESULTS

This study enrolled 147 patients with UADT cancer (116 male and 31 female) and 135 subjects (91 male and 44 female) without cancer. The group with cancer (GC) presented a higher proportion of male population than those without cancer group (NCG) (56% versus 44%). The mean age for group with cancer was 58.4 and range (22-90 years) compared to 51.0 and range (17-90 years) for NCG. Table 1 presents the lifestyle and demographic characteristics between CG and NCG. Significant differences were observed between groups according to gender, first-degree cancer family history, and tobacco and alcohol consumption (Table 1). Also, significant differences among confounding variables for comparison CG and each NCG from hospital, or healthy volunteers (data not shown).

The distribution of the *CCND1* genotypes: GG, GA, and AA were similar in the group with cancer (36.0%, 46.3%, 17.7%) and in the group without cancer (29.6%, 55.1%, 19.3%) (p=0.518). The genotype distribution in the group without cancer was consistent with the Hardy-Weinberg equilibrium (p=0.970), also to group with cancer (p=0.914). Table 2 shows the genotypes frequencies (GG, GA+AA) according to age, gender, tobacco and alcohol use, and cancer first-degree family history. The distribution of tumor stage T and lymph nodes for cases were not statistically different between genotypes GG and (AA+GG) (Table 2).

The logistic regression model adjusted for age as continuous variable, gender, cancer family history, tobacco and alcohol consumers provided non-significant OR for genotype (AA+GA). It was observed OR=0.96 (95% CI: 0.5-1.8, p=0.894) when compared to genotype GG (reference group). The OR calculated to considering each genotype, for GA genotype OR was 1.03 (95% CI: 0.5–2.0, p= 0.923) and for AA, OR=0.79 (95% CI: 0.3-1.8, p= 0.572). Table 3 presents the results of stratified analysis for alcohol non-drinkers, drinkers, non-smokers and tobacco smokers, with estimated OR for combined AA+GA genotypes. A significant OR for AA+GA genotypes OR=7.52 (95% CI: 1.4-39.7, p=0.017) was observed for non-drinkers in an adjusted logistic regression models. No significant risk was found for non-smokers OR=5.38 (95% CI: 0.9-31.4, p=0.061) adjusted for above variables and alcohol use.

Interestingly, for tobacco smokers or alcohol beverage drinking subjects, non-significant risks for combined *CCND1* was obtained in the adjusted analysis.

DISCUSSION

SCC of the UADT is largely associated with environmental factors, especially in individuals with prolonged exposure to tobacco and alcohol (FRANCO et al. 1989; WUNSCH-FILHO 2002). In addition, genetic factors, including deregulation of cell cycle, are also frequently observed in HNSCC (CALLENDER et al. 1995; MULLER et al. 1997). *CCND1* has been suggested to play an important role in

tumorigenesis and progression of HNSCC (CALLENDER et al. 1995; SCULLY et al. 2000b).

In the stratified analysis, for non-drinkers, our data showed high risk for (GA+AA) (OR=7.5) and (OR=3.7) for smokers. Otherwise, in the stratification for non-smokers analysis the *CCND1* (GA+AA) genotypes the OR was 5.4 and a significant OR=11.2 for drinkers were observed. Our results show that for non-smokers, the alcohol drinking is a risk factor for HNSCC and are consistent to the other studies (TALAMINI et al. 1998; FIORETTI et al. 1999), in which they have shown that alcohol consumption is the most important risk factor for oral and pharyngeal cancer in never smokers.

HNSCC is rare in non-exposed individuals and in only few studies they have included enough cancer patients (TALAMINI et al. 1998; FIORETTI et al. 1999). The present study has shown limitations due to small sample size, since each group were not matched by gender, age and tobacco and alcohol habits, and some NCG subjects were not selected in the same basis of CG. Around 70% of our NCG was selected from the same hospital of cases or neighborhood one, and 30% were healthy volunteer individuals. However, the observed *CCND1* genotype distribution and expected from the Hardy-Weinberg equilibrium model suggested no selection bias ($p>0.05$). The frequencies of *CCND1* genotype in our non-cancer group (19.3%, 51.1%, 29.6%) were similar to the observed in the control group from a recent SCCHN study with 16.5%, 49.8%, 26.6% by ZHENG et al. (2001), and also with the control group in a transitional cell carcinoma of urinary bladder cancer study (16.7%, 53.3%, 30.0%) by WANG et al. (2002) in which the NCG was compounded for only healthy subjects.

The most important bias on selecting the NCG is the "recall bias" (WACHOLDER et al. 1992). However, in this study, the major significant variables evaluated are the *CCND1* genotype, alcohol and tobacco consumption and the family history of cancer. The first variable, *CCND1* genotype shows no influence by the "recall bias", the alcohol and tobacco consumption show just a slighted chance of "recall bias", but in the way they are grouped, this chance is even lower. The last variable, cancer family history, can be, but it was assessed only as an adjustment in the model.

A trained interviewer had collected the data of our study, but information about family history and ethnicity were self-reported by each subject. We could not evaluate the ethnicity in the analysis because it is difficult to define the ethnic group in Brazil, due to the high presence of multiracial population.

Non-stratified multivariate analysis was performed including all subjects to assess *CCND1* genotype as a risk factor, but non-significant OR was observed. In a recent study the lack of association was observed between genotype and risk of esophageal SCC (YU et al. 2003). We could not show *CCND1* genotype as a risk factor, but the stratified results have suggested that A allele of *CCND1* polymorphism may be a risk factor especially in individuals without history of alcohol drinking. In the study conducted by ZHENG et al. (2001), the stratified analysis showed high AA genotype adjusted OR=4.8 for non-alcohol drinkers and OR=3.7 for non-smokers and HNSCC. The dominant effect of A allele on tumor progression has been observed in non-small lung cancer and the positive results suggest that a dominant allele A may be a risk factor for tobacco related cancer (BETTICHER et al. 1996). Recently, WANG et al. (2003), has reported a significant

AA genotype risks for *CCND1 A870G* polymorphism to prostate cancer development in relation to GG genotype in Japanese men.

Evaluation of only the main effect of A allele of *CCND1* gene may mask a fundamental interaction with smoking and alcohol drinking, but we did not have enough cancer and non-cancer subjects to allow the estimation of the interaction between smoking and genotype or alcohol consumption and genotype. In this preliminary study, significant differences were verified among confounding variables and NCG from hospital, healthy volunteers and CG, and an uncontrolled residual confounding may raises from the adjusted analysis. In the other hand, we did not observe differences among grouped (AA+GA) and GG genotypes and the study variables, but elevated risks for A allele for non-drinkers, and for non-smokers a non-significant risk was observed and it may be attributed to the small percentages of non-smokers among NCG. However, it is possible that these results obtained from stratified analysis have occurred by chance.

Otherwise, a non-significant OR from stratified analysis for alcohol and tobacco consumers may be that, in the exposed population other genetic factors are involved in activation and detoxifying of chemical carcinogens that may be determinants of susceptibility of SCCHN, whereas the CCND1 genotype may be a determinant in disease progression (CALLENDER et al. 1994; SCULLY et al. 2000b; ZHENG et al. 2001). It is probable that this gene do not act in isolate manner and an evaluation of tobacco and alcohol habits and a multiple genes interaction may be required to understand the association among them (COPPER et al. 1995; SCULLY et al. 2000a e b).

However, prospective epidemiological studies, matched case-control or cohort designed, are needed to establish the exact role of the *CCND1* polymorphism, systemic and topic effects of the presence or absence of tobacco and alcohol and their association with the development of UADT cancers.

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Table 1 - Distribution of cancer and non-cancer subjects according to demographic and lifestyle variables and *CCND1* genotypes.

Variables	Levels	Subjects		
		Non-Cancer	Cancer	
		N=135	N=147	
		Freq.(%)	Freq. (%)	p-value*
Age (years)	≤ 55	80 (59.3)	59 (40.1)	< 0.001
	> 55	55 (40.7)	88 (59.9)	
Gender	Male	91 (67.4)	116 (78.9)	0.029
	Female	44 (32.6)	31 (21.1)	
Race	Caucasian	110 (81.5)	124 (84.4)	0.521
	Non-Caucasian	25 (18.5)	23 (15.6)	
1st.Degree Cancer Family history	No	107 (79.3)	78 (53.1)	<0.001
	Yes	28 (20.7)	69 (46.9)	
Tobacco consumption	Never	63 (46.7)	14 (9.5)	<0.001
	Ex-smoker	39 (28.9)	28 (19.1)	
	Current	33 (24.4)	105 (71.4)	
Alcohol drinking	Never/no	70 (51.9)	19 (12.9)	<0.001
	Ex-drinker	30 (22.2)	47 (32.0)	
	Current	35 (25.9)	81 (55.1)	
CCND1 genotype	GG	40 (29.6)	53 (36.0)	0.518
	GA	69 (51.1)	68 (46.3)	
	AA	26 (19.3)	26 (17.7)	

* p-value obtained by chi-square test

Table 2 - Distribution of *CCND1* genotypes according to variables included in the stratified logistic regression models

Variables	Levels	*CCND1* genotypes GG freq. (%)	GA + AA freq. (%)	p-value
Age	≤ 55	45 (48.4)	94 (49.7)	0.831
	> 55	48 (51.6)	95 (50.3)	
Gender	Male	64 (68.8)	143 (75.7)	0.221
	Female	29 (31.2)	46 (24.3)	
T stage	T1 + T2	18 (34.6)	27 (29.0)	0.486
	T3 + T4	34 (65.4)	66 (71.0)	
Lymph Nodes	Negative	27 (51.9)	45 (47.9)	0.639
	Positive	25 (48.1)	49 (52.1)	
Race	Caucasian	73 (78.5)	161 (85.2)	0.160
	Non-Caucasian	20 (21.5)	28 (14.8)	
1st. Degree cancer family history	No	54 (58.1)	131 (69.3)	0.062
	Yes	39 (41.9)	58 (30.7)	
Tobacco consumption	Never	24 (25.8)	53 (28.0)	0.491
	Ex-smoker	19 (20.4)	48 (25.4)	
	Current	50 (53.8)	88 (46.6)	
Alcohol drinking	Never	26 (28.0)	63 (33.3)	0.221
	Ex-drinker	22 (23.7)	55 (29.1)	
	Current	45 (48.4)	71 (37.6)	

* p-value obtained by chi-square test

Table 3 - Odds ratios estimated from stratified logistic regression adjusted* models

Stratified models	Adjusted OR* (95% CI)	p
Non-drinkers		
(GA + AA) versus GG	7.52 (1.4 – 39.7)	0.017
Smoker (current and ex-smoker)	3.71 (0.9 – 14.5)	0.059
Drinkers		
(GA + AA) versus GG	0.61 (0.3 – 1.3)	0.192
Smoker (current and ex-smoker)	7.12 (2.1 – 24.4)	0.002
Non-smokers		
(GA + AA) versus GG	5.38 (0.9 – 31.4)	0.061
Drinker (current and former)	11.2 (1.6 – 79.2)	0.015
Smokers		
(GA + AA) versus GG	0.77 (0.4 – 1.6)	0.477
Drinker (current and former)	10.1 (3.0 – 34.1)	0.001

* adjusted by gender, first degree cancer family history and age as continuous variable

COMENTÁRIOS

5 COMENTÁRIOS

Alguns polimorfismos de DNA têm sido descritos nos genes codificadores das enzimas envolvidas no metabolismo de carcinógenos do tabaco ou do álcool (etanol). Muitos desses polimorfismos têm sido examinados como possíveis biomarcadores para risco do desenvolvimento de cânceres relacionados ao tabaco ou ao álcool, mas os resultados ainda são controversos (FURBERG e AMBROSONE 2001).

Alguns estudos verificaram o polimorfismo do *ADH3* na posição Ile349Val como possível fator de risco no desenvolvimento de neoplasias. FREUDENHEIM et al. (1999), obtiveram resultados onde o polimorfismo do gene do *ADH3* estava relacionado a um aumento de risco para o desenvolvimento de câncer de mama em mulheres em pré-menopausa, mas não foram significativos para as pós-menopausadas. Para os adenomas colo-retais, o estudo de TIEMERSMA et al. (2003) também encontrou aumento significativo nos riscos para o desenvolvimento da neoplasia estudada na presença do polimorfismo do gene do *ADH3*. Os resultados obtidos por OLSHAN et al. (2001), não mostraram o polimorfismo do *ADH3* como fator de risco para as neoplasias malignas de cabeça e pescoço. Entretanto, a análise estratificada do nosso estudo indica o genótipo AA como sendo um possível fator de risco para o câncer de VADS em indivíduos que consomem baixas quantidades de bebidas alcoólicas.

Embora os polimorfismos envolvidos no metabolismo de carcinógenos tenham importante papel no desenvolvimento das neoplasias malignas, as alterações

nos genes envolvidos no controle do ciclo celular são potenciais candidatos na suscetibilidade ao câncer. A alteração nos genes do *p53, H-ras1, CCND1* e *p21* têm apresentado riscos moderados ou elevados para o desenvolvimento de neoplasias malignas de VADS, embora as casuísticas consideradas nos estudos tenham sido pequenas (FURBERG e AMBROSONE 2001).

No estudo realizado por WANG et al. (2002) os indivíduos portadores do alelo A do gene *CCND1* mostraram elevado risco para o desenvolvimento de câncer de bexiga. Aumento de risco também foi observado para o polimorfismo da ciclina D1 em câncer colo-retal, conforme resultados obtidos por KONG et al. (2001). Em um recente estudo tipo caso-controle desenvolvido em Houston nos Estados Unidos, cuja casuística consistiu-se de 233 casos de câncer de cabeça e pescoço e 248 indivíduos saudáveis (controles), o genótipo AA da ciclina D1 resultou num relativo aumento de risco, cerca de 2 vezes de se desenvolver câncer de cabeça e pescoço e, 4 vezes para os indivíduos não fumantes e de 5 para os que não consomem bebidas alcoólicas (ZHENG et al. 2001). O nosso estudo não demonstrou diferenças significativas nas estimativas dos riscos para o polimorfismo do gene da *ciclina D1* no desenvolvimento do câncer de VADS em análise não estratificada. Entretanto, mostrou riscos aumentados em 5 vezes para os não fumantes e em 7 para os que não consomem bebidas alcoólicas, para o alelo A do genótipo, em análise estratificada de dados. Devido a nossa pequena casuística de pacientes com câncer que não consomem bebidas alcoólicas, os indivíduos com o genótipo AA foram agrupados aos do grupo com o genótipo heterozigoto GA. O polimorfismo G870A no éxon 4 do gene *CCND1* foi considerado nos dois estudos.

Embora os resultados das pesquisas realizadas ainda sejam controversos, o presente estudo indica que a presença do alelo A do polimorfismo do gene da ciclina D1 (*CCND1*) e o genótipo AA do *ADH3*, podem ser candidatos a fatores de risco para o desenvolvimento de câncer de VADS em indivíduos com baixa ou nenhuma exposição aos carcinógenos do tabaco e ou álcool.

Para se avaliar a possível associação das neoplasias malignas e a atividade ou possíveis alterações de alguns genes, os estudos epidemiológicos de coorte ou caso-controle vêm sendo adotados (FURBERG e AMBROSONE 2001). Entretanto, com os avanços da genética e a utilização de modernas técnicas de *microarrays*, os desenhos de estudos da epidemiologia molecular devem ser adequadamente delineados, considerando-se a seleção dos grupos de indivíduos, modelos matemáticos e as análises estatísticas (FURBERG e AMBROSONE 2001). A diversidade nos resultados obtidos dos vários estudos epidemiológicos tipo caso-controle, onde objetivam a verificação dos polimorfismos como fatores de risco, é explicado, em parte, devido à variada seleção dos indivíduos que compõem o grupo controle (INFANTE-RIVARD 2003).

A suscetibilidade associada a genes de baixa penetrância, como os que estão envolvidos no metabolismo, reparo de DNA e ciclo celular, têm menos impacto no risco de desenvolvimento de câncer, entretanto podem apresentar elevados riscos atribuíveis (STURGIS e WEI 2002). Os resultados de estudos, verificando a suscetibilidade genética ainda são inconsistentes, provavelmente devido às metodologias e desenhos de estudo adotados (FURBERG e AMBROSONE 2001).

Muitos polimorfismos estão sendo abordados na pesquisa molecular. Os genes envolvidos na fase I, onde ocorre a presença ou ativação de enzimas oxidativas

tais como a família dos citocromos *P450* e do *ADH* e, os da fase II do processo metabólico onde ocorre a detoxificação que geralmente inativa esses genotóxicos, como as glutationas S-transferase (GSTs) e as aldeído desidrogenases (ALDH) têm mostrado serem possíveis fatores de risco para o câncer de VADS (BARTSCH et al. 2000). Em geral, as maiorias dos agentes químicos carcinogênicos não produzem os seus efeitos biológicos, mas requerem ativação metabólica de outras enzimas e, é provável que haja interações entre algumas dessas enzimas metabolizantes carcinógenas, que são encontradas no tabaco ou nas bebidas alcoólicas, e essas interações tenham um importante papel no desenvolvimento do carcinoma espinocelular de VADS como no caso dos CYPs, GSTs, NATs e outros (HARRIS 1987; HAYASHI et al. 1991; KATO et al. 1994; HAYES e PULFORD 1995; JANKHE et al. 1996; HUNG et al. 1997; JOURENKOVA et al. 1998; JOURENKOVA-MIRONOVA et al. 1999a e b; HEIN et al. 2000; OLSHAN et al. 2000).

Para que a interação genótipo, fenótipo e exposição, incluindo polimorfismos das fases I e II possam ser verificados, a realização de estudos epidemiológicos do tipo caso-controle incluindo adequada casuística, onde os controles sejam devidamente selecionados como sendo de base hospitalar ou populacional, com grande número de casos sejam delineados e conduzidos (FURBERG e AMBROSONE 2001). As associações entre os genes envolvidos com metabolismo, ciclo celular, reparo de DNA e imunidade, com o risco para o desenvolvimento de neoplasias malignas das VADS vêm sendo verificadas separadamente nos estudos epidemiológicos (STURGIS e WEI 2002).

Contudo, com as novas técnicas da genética disponíveis, estudos combinando os vários genótipos de várias regiões polimórficas, onde os vários genes podem ser analisados simultaneamente, devem ser considerados na pesquisa epidemiológica do câncer (STURGIS e WEI 2002).

Outros agentes ambientais além do tabaco e álcool também devem ser avaliados (STURGIS e WEI 2002). O consumo de etanol tem sido associado à inadequada nutrição e dieta alimentar, deficiente de elementos protetores ao desenvolvimento do câncer de VADS (SIMANOWSKI et al. 1995).

Embora este estudo apresente deficiências em seu delineamento devido à inclusão indiscriminada de indivíduos componentes do grupo controle, dois polimorfismos foram avaliados, sendo um deles relacionado ao metabolismo de carcinógenos e o outro ao controle do ciclo celular. O fato de não termos obtido riscos significativos em análise não estratificada de dados, o que sugere a necessidade da inclusão de outros conhecidos fatores de risco para o desenvolvimento das VADS, como o consumo de frutas e vegetais na abordagem estatística. Por outro lado, outros genes possivelmente estão interagindo com os aqui estudados, como o polimorfismo do *ALDH*. Futuros estudos verificando as combinações entre os genótipos do *ADH3* e *ALDH2*, ou com outros genes álcool ou tabaco-associados, podem ser informativos sobre a carcinogênese. Para que essas interações gene-gene sejam possíveis, é necessária uma maior casuística em cada grupo comparação.

As análises estratificadas deste estudo, que incluiu a participação de reduzido número de indivíduos com baixa ou nenhuma exposição aos carcinógenos, mostraram elevados riscos de indivíduos portadores dos polimorfismos dos genes

ADH3 ou *CCND1* virem a desenvolver uma neoplasia de VADS, indicando a necessidade da realização de futuros estudos para a confirmação, ou não, destes achados.

CONCLUSÕES

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1. O genótipo AA do polimorfismo do *ADH3* mostrou ser um possível fator de risco em indivíduos que consomem baixas quantidades de tabaco.

2. O genótipo homozigoto A do polimorfismo do *ADH3* mostrou ser um possível fator de risco em indivíduos que consomem baixas quantidades de álcool.

3. Os resultados sugerem que a presença do alelo A do polimorfismo da *CCND1* confere riscos elevados, especialmente para indivíduos sem história de consumo de bebidas alcoólicas.

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ANEXOS

Anexo 1 Figuras representativas das análises do polimorfismo no gene da *ciclina D1*

Cromatogramas representativos da eluição do fragmento amplificado e das seqüências do gene da *ciclina D1*, mostrando os perfis homozigoto ou heterozigoto que contem o polimorfismo. As metodologias utilizadas estão descritas nos artigo 1 (polimorfismo do ADH3) e artigo 2 (polimorfismo da *CCND1)*.

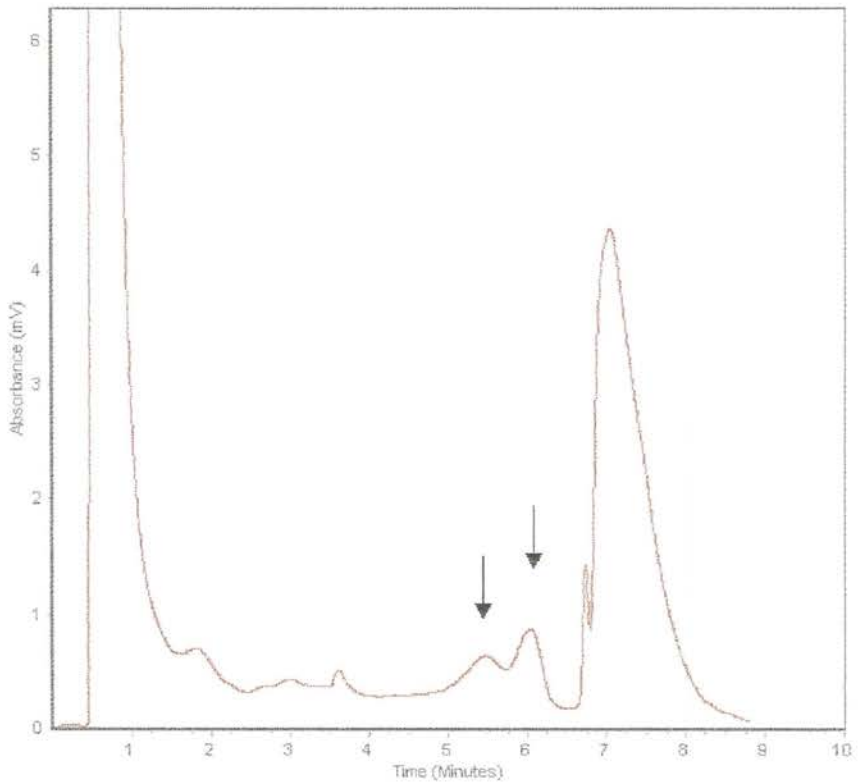

Figura 1 Cromatograma representativo da eluição do fragmento amplificado do gene, mostrando um perfil heterozigoto.

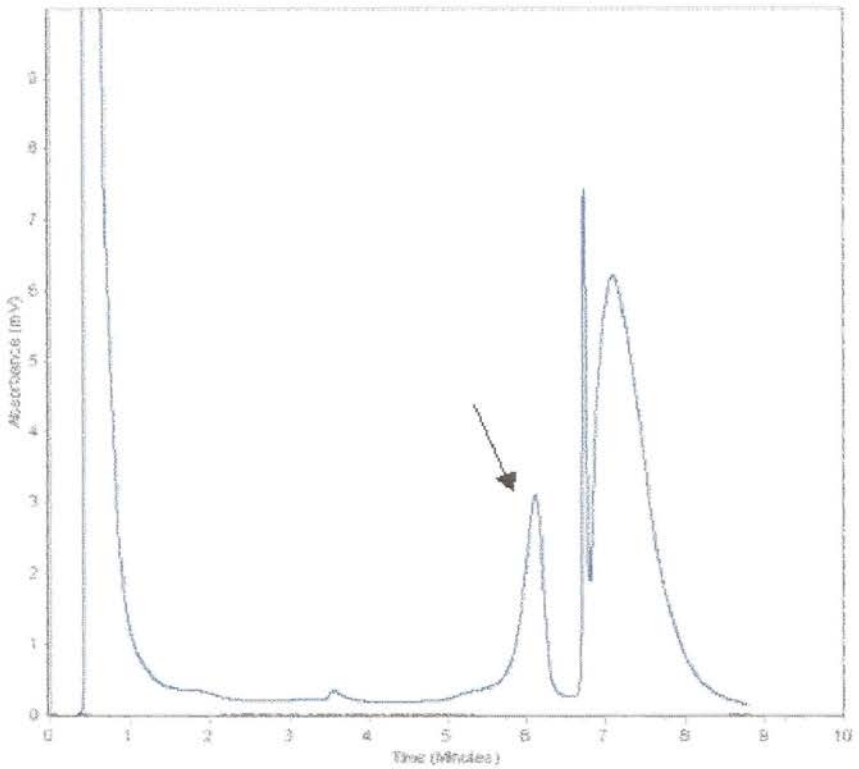

Figura 2 Cromatograma representativo da eluição do fragmento amplificado do gene, mostrando um perfil homozigoto do paciente 133TM

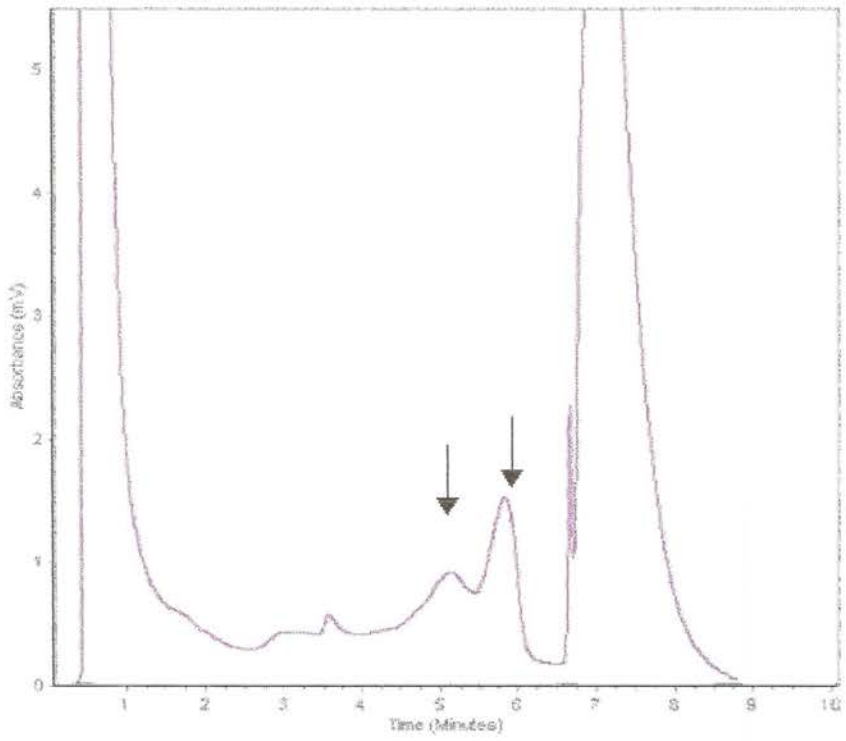

Figura 3 Cromatograma representativo da eluição do fragmento amplificado do gene, mostrando um perfil heterozigoto, na análise da mistura contendo amostra do paciente 133TM com controle de genótipo AA.

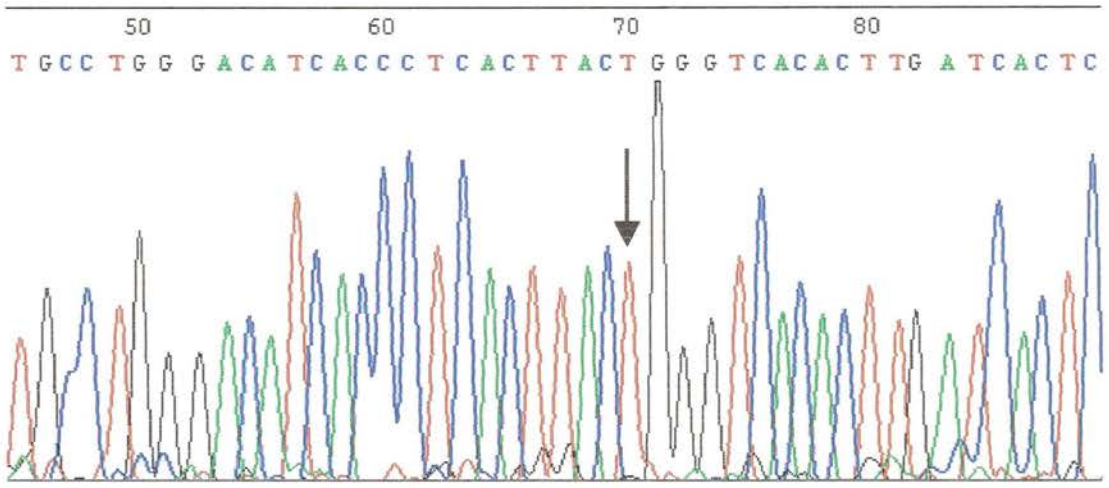

Figura 4 Seqüência de DNA , Genótipo AA (homozigoto para o polimorfismo)

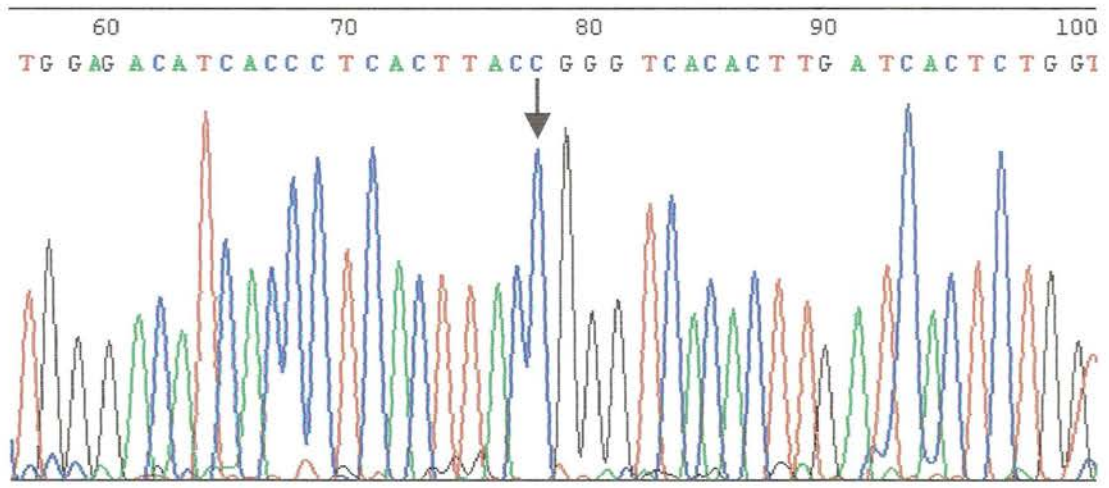

Figura 5 Seqüência de DNA, Genótipo GG (homozigoto selvagem)

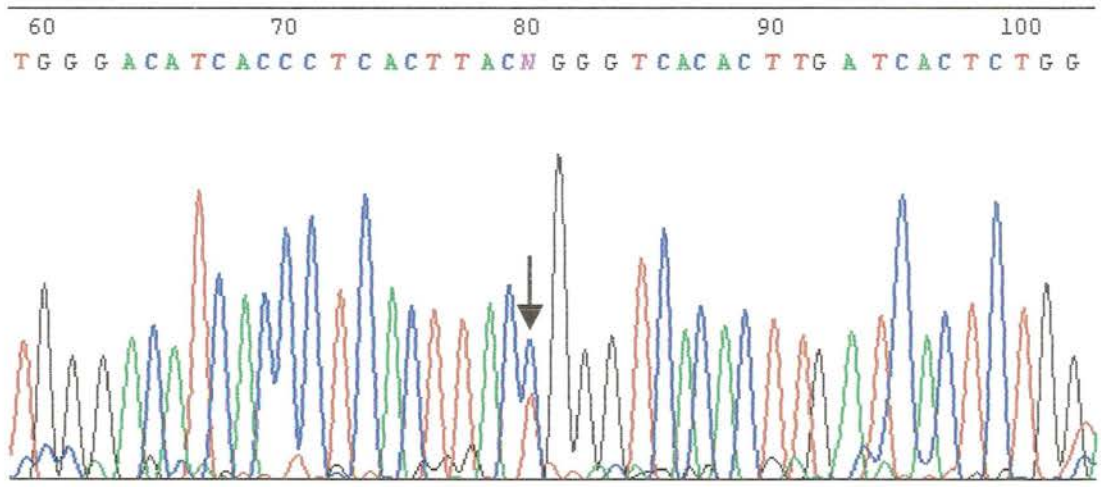

Figura 6 Seqüência de DNA, Genótipo GA (heterozigoto)

Anexo 2 Estimativa dos o*dds ratios* (OR) obtidos através da análise de regressão logística não estratificada.

Polimorfismo	OR ajustado* (95% IC)	p
CCND1		
(GA + AA) versus GG	0,94 (0,5 – 1,7)	0,851
consome tabaco	5,43 (2,3 – 12,7)	<0,001
consome álcool	8,98 (3,3 – 24,1)	<0,001
ADH3		
AA versus (GG + GA)	0,73 (0,4 – 1,3)	0,292
consome tabaco >25 packyears	2,43 (1,3 – 4,6)	0,007
consome álcool > 100 kg etanol	8,28 (3,9 – 17,4)	<0,001

* ajustado por gênero, idade (≤55; >55 anos), história familiar de cancer

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RE: MS # OOA30106, ALCOHOL DEHYDROGENASE 3 GENOTYPE AS A RISK FACTOR FOR UPPER AERODIGESTIVE TRACT CANCERS

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