FRANCISCO CARLOS AMANAJÁS DE AGUIAR JÚNIOR

ANÁLISE CLINICOPATOLÓGICA E IMUNOHISTOQUÍMICA DE CARCINOMAS ESPINOCELULARES BUCAIS EM PACIENTES COM RECIDIVA LOCAL PRECOCE.

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Estomatopatologia. Área de Estomatologia.

PIRACICABA

2006

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Área de Estomatologia.

Orientador: Prof. Dr. Luiz Paulo Kowalski
Co-orientador: Prof. Dr. Oslei Paes de Almeida

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SUMÁRIO

RESUMO

Neste trabalho, foram analisados os dados clinicopatológicos e a imunoexpressão de Ki-67, p53, bcl-2, FAS, Erb-B2, E-caderina e β-catenina em 27 carcinomas espinocelulares bucais (CEBs) de pacientes acometidos por recidiva local precoce, comparando-os com um grupo controle constituído por 54 pacientes com CEB sem história de recorrência, portadores de tumores de mesma localização e estadiamento clínico. Foram selecionados para este estudo 81 pacientes submetidos à cirurgia como primeiro tratamento no Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia do Hospital do Câncer, São Paulo/SP. Adicionalmente, em 17 CEBs foi realizada a comparação da imunoexpressão de Ki-67, p53, FAS, Erb-B2, E-caderina e β-catenina com suas respectivas recidivas locais precoces. Dentre todas variáveis analisadas, diferenças significativas no relato do consumo de álcool, na abordagem terapêutica instituída e na imunoexpressão de Ki-67, FAS e β-catenina (p=0.028, p=0.005, p=0.026 respectivamente) foram observadas. Uma fraca associação na imunomarcação por Ki-67, Erb-B2 (membrana), E-cadherin e β-catenin (ambos citoplasma) entre os CEBs e suas respectivas recidivas locais foi demonstrada, (índice kappa= 0.179, 0.337, 0.442 e 0.326 respectivamente). Os resultados obtidos sugerem que a imunoexpressão de Ki-67, FAS e β-catenina são potenciais marcadores do risco de desenvolvimento de recorrência local precoce do CEB e que a expressão de alguns biomarcadores analisados se altera nas recorrências locais.

ABSTRACT

In this study, the clinicopathological data and the immunoexpression of Ki-67, p53, bcl-2, FAS, Erb-B2, E-cadherin and β-catenin were analyzed in 27 oral squamous cell carcinoma (OSCC) of patients with early local recurrence compared with a control group composed by 54 patients with non-recurrent OSCC. All the patients in both groups were selected according to the tumor location and the clinical stage. The 81 patients assigned for this study had undergone surgery as the primary treatment at the Department of Head and Neck Surgery and Otorhinolaryngology, A.C. Camargo Cancer Hospital, São Paulo, Brazil. Additionally, a comparison was made between the immunoexpression of Ki-67, p53, bcl-2, FAS, Erb-B2, E-cadherin and β-catenin, and the early local recurrences in 17 cases. Among the variables studied, significant differences were observed in the report of alcohol consumption, the therapeutic assessment adopted, and the immunoexpression of Ki-67, FAS, Erb-B2 and β-catenin (p=0.028, p=0.005, p=0.026, respectively). There was no significant association in the immunoexpression of Ki-67, Erb-B2 (membrane), E-cadherin e β-catenin (both cytoplasmic) between the OSCCs and their corresponding early local recurrences (kappa index =0.179, 0.337, 0.442 e 0.326, respectively). The results obtained in this study suggest that the immunoexpresion of Ki-67, FAS e β-catenin are potential markers for the risk of early local recurrence, and thus the expression of some biomarkers analyzed changed in the recurrent tumor.

1. INTRODUÇÃO

Dentre os tumores malignos que acometem a cavidade bucal, o carcinoma espinocelular (CEC) é o que tem mais frequente, representando cerca de 95% de todas as neoplasias malignas desta região. O CEC de língua é a lesão maligna intra-bucal mais comum e representa 40% dos carcinomas de boca (LEVY *et al.* 1991, REGEZI *et al*., 1999, NEVILLE *et al*., 2002). Uma maior incidência é relatada em pacientes acima dos 40 anos, e em indivíduos do gênero masculino. No entanto, tem-se notado um aumento da incidência em adultos jovens bem como em mulheres (SAPP *et al.*, 1997).

O carcinoma espinocelular bucal (CEB) representa um sério problema de saúde pública no mundo, com tendência a crescimento particularmente nos países em desenvolvimento (SANKARANARAYANAN *et al*., 1998). No Brasil, o CEB corresponde de 3 a 5% do total das neoplasias malignas e está entre os dez tipos mais comuns de câncer.

Alguns poucos dados foram acrescentados sobre a possível etiologia e mecanismos relacionados á sua carcinogênese (JEFFERIES & FOULKES, 2001). Apesar do grande número de fatores citados no desenvolvimento do CEB, a associação do álcool e tabaco e radiação ultravioleta (no caso do CEC de lábio), são os únicos fatores de risco evidentes (SCULLY, FIELD, TANZAWA, 2000a; SCULLY, FIELD, TANZAWA, 2000b). Os agentes carcinogênicos ou pró-carcinogênicos atuam no epitélio normal, causando mutações no DNA. O acúmulo destas mutações altera grupos de genes que desempenham funções

características na célula, são estes: os proto-oncogenes, os genes supressores de tumor, as telomerases e os genes que regulam o processo de apoptose (SCULLY, FIELD, TANZAWA, 2000b; SCULLY, FIELD, TANZAWA, 2000c; PETRUZZELI, 2001).

O CEB exemplifica as teorias de cancerização de campo e do processo de carcinogênese "multistep" (TAE *et al.*, 2000; BRAAKHUIS *et al.,* 2004). O termo "campo de cancerização" foi primeiramente utilizado por SLAUGHTER *et al*, em 1953, no entanto, os autores não proporcionaram uma clara definição, e através de exame histopatológico foram descritas algumas características importantes sobre este fenômeno: (1) o carcinoma bucal se desenvolve em múltiplas áreas multifocais de modificação pré-cancerosa (que muitos interpretam como sendo de múltiplos eventos independentes), (2) tecido alterado ou displásico circunda o tumor, (3) o carcinoma bucal consiste de múltiplas e independentes lesões que às vezes coalescem, (4) a persistência de tecido alterado ou displásico explica a presença de ocorrência de segundos tumores primários e recidivas. A interpretação de que pequenos e independentes focos cancerosos coalescem em um único tumor é questionável. No entanto, o conceito de que alterações precancerosas se extendem além da área macroscopicamente vísivel do tumorr é bastante válida (BRAAKHUIS, 2002).

Os ensaios terapêuticos estão estratificados em sua maioria pelo estadiamento clínico (segundo a Classificação dos Tumores Malignos (TNM) proposta pela União Internacional Contra o Câncer (UICC)). O tratamento do CEB está baseado na cirurgia ou radioterapia nos estádios clínicos iniciais e na

associação de ambas nos estádios clínicos avançados, restando para a quimioterapia apenas os protocolos de preservação de órgão, adjuvância à radioterapia ou paliação (PARISE, CARVALHO & KOWALSKI, 1998).

Em geral, a sobrevida de 5 anos chega a cerca de 45 a 50% (CARVALHO, MAGRIN, KOWALSKI, 2003). O prognóstico declina com o aumento de tamanho e estádio clínico do tumor, sendo o diagnóstico precoce e tratamento adequado essenciais para previnir morte prematura, desfiguramento estético e sequela funcionais.

Como mencionado, o tratamento e prognóstico desta doença estão geralmente baseados no estadiamento clínico e em alguns parâmetros histopatógicos (SIECZKA, 2001; CARVALHO, DIAS & CABRAL, 2003). Entretanto, nem sempre a evolução clínica segue o padrão clínicopatológico esperado.

Apesar de avanços na terapia, a sobrevida dos pacientes tratados não tem aumentado significantemente nestas últimas duas décadas, uma importante razão para este fato está na relativa alta taxa de recorrência observada nos pacientes acometidos (KOWALSKI *et al.*, 1993; SHIN *et al.*, 1996; WOOLGAR,1999; KHURI *et al.*, 2000; TABOR *et al.,* 2001; FUNK *et al.*, 2002; CARVALHO, MAGRIN, KOWALSKI, 2003).

De uma forma geral, o CEB apresenta um risco intermediário de recorrência e sua causa tem sido relacionada com diversas características clínicopatológicas como: estadiamento TNM, grau de diferenciação histopatológica, espessura tumoral, invasão vascular e perineural, e comprometimento das margens cirúrgicas ressecadas (CHEN *et al.*, 1987,

ZELEFSKY *et al*., 1993; HORIUCHI *et al*., 1993; WILLIAMS *et al*., 1994; TEIXEIRA, *et al*. 1996; BONGERS *et al*., 1996; FAGAN *et al*., 1998; MISHRA, 1999; AL-RAHJI *et al*., 2000; PARTRIGDE *et al.,* 2000, NIIMI *et al*., 2001).

A recidiva local do tumor primário é um problema significativo no tratamento e tem um impacto negativo na sobrevida (SARKARIA & HARARI, 1994; SHIN *et al*., 1996; OKURA *et al*., 1997; BRAAKHUIS *et al*., 2002). Esta ocorre em cerca de 10-30% dos casos avançados, mesmo naqueles que histopatologicamente possuíam margens cirúrgicas livres após a ressecção (BRENNAN *et al*., 1995; HICKS *et al*., 1997; TABOR *et al*., 2001).

As recidivas locais ocorrem até 5 anos após o tratamento inicial, a maioria deles antes de 2 anos. Recorrências que ocorrem precocemente são raramente tratáveis e de mau prognóstico (AGRA *et al,* 2005).

A literatura carece de trabalhos que associe dados clínicos, histopatológicos no intuito de elucidar os fatores envolvidos na recorrência precoce do carcinoma espinocelular bucal depois de adequada terapia.

Marcadores Imunohistoquímicos

Existem muitos estudos, mas poucos marcadores prognósticos de agressividade definidos para carcinomas de cabeça e pescoço (JEFFERIES & FOULKES, 2001; GINOS *et al* 2004). Na literatura são poucos estudos que correlacionam especificamente alterações e variações na expressão protéica através de imunohistoquímica em carcinomas espinocelulares bucais com recidiva

local, particularmente naqueles que casos onde a recidiva ocorre precocemente ou de forma agressiva.

p53

O gene TP53 é um gene supressor de tumor localizado no braço curto do cromossomo 17 e o produto da sua expressão, a proteína p53, funciona como inibidor da divisão celular na fase G1 do ciclo celular, podendo induzir apoptose. A proteína p53 atua como um fator de transcrição para várias outras proteínas e tem como função manter a integridade do material genético celular. Ao sinal de falha no DNA celular, ativa a transcrição de p21 (Waf1/cip1) (proteína de 21 kDa) que mantém a célula em G1, permitindo a checagem do DNA e a atuação dos mecanismos de reparo do DNA. Se estes mecanismos falharem ou não forem capazes de corrigir os erros do DNA, p53 aciona os mecanismos para que haja a morte celular programada (AGARWAL *et al.,* 1999). Em células normais, a proteína p53 selvagem ou natural atua é rapidamente degradada (meia vida de 20 minutos), não se acumulando em níveis detectáveis por imunohistoquímica. As mutações no gene TP53 levam à formação de proteínas alteradas com meia vida de 6 a 8 horas, que se acumulam nas células neoplásicas e podem ser detectadas por imunohistoquímica (BRAITHWAITE, ROYDS & JACKSON, 2005).

As mutações do gene TP53 são comumente relatadas no CEB, e sua participação na progressão tumoral bem como seu valor prognóstico, são exaustivamente estudados, principalmente pelo acúmulo da proteína

correspondente. No entanto, não há um consenso na literatura atual com relação à freqüência de alterações do gene TP53 encontrados em CEBs, as maiores dificuldades na interpretação destes dados reside em diferenças na amostra e técnicas utilizadas (PARISE, CARVALHO & KOWALSKI, 1998; ALMEIDA *et al.,* 1999; KOONTONGKAEW *et al.,* 2000; SCULLY, FIELD, TANZAWA 2000; KOELB *et al*., 2001; PANDE *et al.,* 2002, VORA *et al.*,2003) .

Com relação ao seu valor prognóstico associado à recorrência local, SHIN *et al.* (1996) avaliaram a imunoexpressão da proteína p53 como elemento preditivo tanto da recorrência do primeiro tumor como do aparecimento de segundo tumor primário (stp) em 61 pacientes com câncer de cabeça e pescoço, previamente tratados. Detectaram a expressão de p53 nas células tumorais de 41 pacientes (59%), o que estava associado a um prognóstico adverso para a sobrevida, para o tempo de recidiva do tumor inicial e para o tempo de aparecimento do stp. Concluíram que o tempo para o aparecimento de falhas do tratamento inicial após definitiva terapia local radical foi significativamente encurtado no grupo positivo para o p53 comparado com o grupo negativo para o p53.

Bcl-2

A proteína Bcl-2 reside predominante na membrana externa da mitocôndria, na membrana do retículo endosplasmático e na membrana nuclear, atuando na

inibição dos mecanismos de apoptose. A habilidade da proteína Bcl-2 em inibir a apoptose sem afetar a proliferação celular, definiu o gene Bcl-2 como uma importante categoria de oncogene. A despeito de ser expressa e detectada pela imunohistoquímica, esta proteína parece não influenciar o prognóstico ou a sobrevida dos pacientes com CEB (XIE *et al.*,1999 ; YUEN *et al.,*2002), não havendo relatos da possível relação desta proteína no comportamento tumoral de CEBs que recidivam precocemente.

Ki-67

A proteína Ki-67 é uma proteína nuclear, cuja expressão aumentada relaciona com aumento da proliferação celular, com pico de expressão na fase M e conseqüente diminuição após a mitose (MATSUMURA *et al*., 1989; CORDON-CARDO, 1995; SCHOLZEN & GERDES, 2000). Desta forma, somente as células em proliferação expressam Ki-67, ao contrário das células quiescentes (G0), que não produzem esta proteína. Anticorpos contra Ki-67 têm mostrado grande valor em estudos de biologia tumoral, pois parecem marcar a fração proliferativa dos tecidos normais e neoplásicos (KILL, 1996; SHOLZEN & GERDES, 2000). Apesar de bastante utilizado como marcador de proliferação celular, a sua função biológica ainda é desconhecida e sua utilização como marcador molecular para o estabelecimento prognóstico do CEB é ainda controverso (SITTEL *et al.,*1999; RAYBAUD *et al.,*2000, KOELB *et al.,* 2001; COUTORE *et al.,* 2002).

ÁCIDO GRAXO SINTASE

A ácido graxo sintase (FAS) é um complexo multifuncional composto de sete enzimas que são requeridas na síntese endógena de ácidos graxos saturados de cadeia longa, a partir dos precursores acetil-CoA e malonil-CoA (STOOPS & WAKIL, 1981).

Células normais preferencialmente utilizam ácidos graxos circulantes oriundos da dieta para suas demandas funcionais. No entanto, tem sido reportado que células tumorais expressam altos níveis de FAS e usam uma via endógena de produção de ácidos graxos. A produção anormal de FAS pode ser um reflexo de um descontrole do ciclo celular, ou seja, uma alta atividade proliferativa aumentaria as necessidades de ácidos graxos para a síntese das membranas celulares das células em divisão (KUHAJDA *et al.* 1994). Alguns estudos demonstram que diversos tumores possuem uma expressão aumentada de FAS, e que tal fato está diretamente correlacionado com um prognóstico desfavorável (PIZER et al., 1998; VISCA et al., 1999; KUHAJDA,2000).

Existem na literatura poucos trabalhos que estudaram a expressão de FAS em CEBs. KRONTIRAS *et al.*, 1999, observaram que havia relação entre um prognóstico favorável e carcinomas espinocelulares de língua que expressavam intensamente FAS. SILVA *et al.*, 2004. não constataram qualquer relação entre a expressão de FAS e prognóstico, nos CEBs estudados.

E-CADERINA

As caderinas pertencem a uma grande família de moléculas de adesão intercelular, essenciais para a manutenção da coesividade celular. No grupos das caderinas clássicas encontram-se: E-caderina, N-caderina, P-caderina e outras. No grupo das caderinas desmossomais estão as desmogleínas e as desmocolinas. Dentre todas citadas, a Caderina E é glicoproteína integral de membrana cálcio dependente que forma complexos moleculares com outras proteínas de adesão citoplasmáticas denominadas cateninas (RAMBURAM & GOVENDER, 2002, WONG & GUMBINER, 2003)

A caderina E é a principal molécula de adesão do epitélio e está implicada na carcinogênese por se encontrar suprimida numa grande variedade de cânceres humanos (MARGULIS et al., 2005). O restabelecimento dos complexos juncionais em linhagens celulares neoplásicas resulta na reversão de um fenótipo invasivo para um fenótipo epitelial benigno (KUDO et al.,2004).

CHANG *et al*., 2002, estudaram a expressão da E-caderina em 109 CEC em língua (93 tumores primários, 7 recorrências locais e 9 metástases para linfonodos) através de imunohistoquímica e observaram a perda de expressão desta em 83% dos tumores primários, 86% das recidivas e 89% nas metástases. Os autores concluíram que a perda de expressão da caderina-E está relacionada a um prognóstico desfavorável.

β- CATENINA

A **β-**catenina foi originalmente isolada como uma proteína associada à região citoplasmática da caderina E, e possui um papel fundamental na adesão celular mediada por cálcio, sendo ainda, objeto de intensa investigação científica (BARTH *et al.,* 1997). A **β-**catenina exibe duas funções distintas. Primeiramente, na junção celular, sendo essencial para a promoção da adesão intercelular, através da conexão da caderina E com o citoesqueleto de actina. Sua segunda função, quando na forma livre, sendo que uma vez ligada ao fator de transcrição celular, migra para o núcleo onde atua na atividade transcripcional, com atuação em vários genes alvo, entre eles a ciclina D1(MIGUEL & AMORIM, 2004). Mutações da **β-**catenina parecem ser um passo crucial na transformação maligna, sugerindo um papel importante no controle da proliferação celular ou mesmo apoptose (KUDO *et al*..,2004). Estudos acerca das funções desta proteína atestam sua multifuncionalidade pois combina características de proteína de adesão nas junções intercelulares, bem como de fator de transcrição através de diferentes vias de sinalização (BAGUTTI, SPEIGHT AND WATT, 1998; KUDO *et al*., 2000; WONG & GUMBINER, 2003).

LO MUZIO *et al.* (1999) analisaram por imunohistoquímica a expressão de **β-**catenina em 30 casos de carcinomas bucais com diferentes graus de diferenciação. Verificaram que a expressão destas proteínas mostrou uma relação inversa ao grau de diferenciação, sendo a expressão na membrana reduzida em neoplasias mais agressivas.

CHOW *et al.* (2001) observaram uma significativa redução na expressão das cateninas em carcinomas de células escamosas na língua, linfonodos metastáticos e tumores recidivantes, o que sugere uma possível correlação da redução na expressão desta proteína e recorrência tumoral.

Apesar de trabalhos terem demonstrado uma redução na expressão desta proteína em CEB, a importância clínica desses achados ainda é controversa (BAGUTTI, SPEIGHT & WATT, 1998).

ErbB2

O oncogene ErbB2 codifica um receptor de fator de crescimento transmembrana de 185 kD que desempenha importante papel na diferenciação, desenvolvimento e sinalização mitogênica em células normais, cuja expressão aumentada tem sido relacionada ao comportamento agressivo de alguns tumores (HOLBRO,CIVENNI & HYNES,2003). Tal receptor apresenta grande homologia com o receptor do *Epidermal Growth Factor* (EGFR). O EGFR possui uma função importante na mitogênese epitelial, e diversos estudos mostram que este receptor está com expressão aumentada em CECs de cabeça e pescoço (CHRISTENSEN, 1998; O-CHAROENRAT, 2002).

Os estudos em CECs de cabeça e pescoço, incluindo diferentes localizações, não foram conclusivos sobre o valor prognóstico do Erb-B2, embora este estivesse superexpresso entre 40% e 60% dos carcinomas analisados (PARISE, CARVALHO & KOWALSKI, 1998; SHIGA *et al.,* 2000; VORA *et al.,* 2003; SILVA *et al.,* 2004; ULANOVSKI *et al.*, 2004).

2. OBJETIVOS

Os objetivos deste estudo foram:

2.1. Comparar os dados clinicopatológicos e a expressão imunohistoquímica de Ki-67, p53, bcl-2, FAS, Erb-B2, E-caderina e β-catenina em 27 carcinomas espinocelulares bucais (CEBs) de pacientes acometidos por recidiva local precoce, com um grupo controle constituído por 54 pacientes com CEB, sem história de recidiva, de mesma localização e estadiamento clínico.

2.2. Comparar em 17 casos de CEBs a imunoexpressão de Ki-67, p53, FAS, Erb-B2, E-caderina e β-catenina com suas respectivas recidivas locais precoces.

3. CAPÍTULO 1

CLINICOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF ORAL SQUAMOUS CELL CARCINOMA IN PATIENTS WITH EARLY LOCAL RECURRENCE: A COMPARATIVE STUDY.

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SUMMARY

Early local recurrence is one of the main causes of treatment failure after definitive therapy of oral squamous cell carcinoma (OSCC), contributing significantly for the relative low survival rates of this neoplasia. The aim of this study was to investigate the clinical, histological and molecular factors involved in early local recurrence of OSCC, which may lead to better risk assessment in particular cases. Patients and Methods: 27 patients with early local recurrent (ELR) OSCC were matched with 54 patients with the same clinical stage and tumor site but without local recurrence, in a pair-matched study design. All cases were evaluated in relation to the clinicopathological features and immunohistochemical expression of Ki-67, p53, bcl-2, FAS, Erb-B2, β-catenin, and E-cadherin. The variables associated with ELR were alcohol consumption (p=0.019), treatment performed (p=0.041), and expression of Ki-67 (p=0.028), FAS (p=0.005) and membrane β-catenin (p=0.026). The multivariate survival analysis (Cox regression) showed that surgery with adjuvant radiotherapy [OR=0.26 (95% CI, 0.1-0.6)] and FAS expression [OR=0.21 (95% CI, 0.1-0.5)] had a significant effect for ELR development. Therefore, both treatment and tumoral molecular characteristics seem to be involved in early local recurrence.

KEYWORDS: oral cancer, immunohistochemistry, treatment failure, local recurrence, oral squamous cell carcinoma.

INTRODUCTION

Squamous Cell Carcinoma is the most common malignant neoplasm of the oral cavity[1]. Oral Squamous Cell Carcinoma (OSCC) is identified as a significant public health threat all over the world[2]. The management of OSCC varies considerably, but the standard procedure includes surgery with or without neck dissection, followed by adjuvant radiotherapy[3]. Despite all efforts and therapeutic developments, the 5-year survival rate for head and neck cancers has not remarkably improved over the last two decades [2,4]. In clinical practice, treatment planning and prognosis for patients with OSCC is mainly based on the TNM classification[5]. However, the clinical outcome does not always follow the expectation of those parameters, indicating that other factors related to the patient or tumoral biological characteristics can be relevant.

The rates of OSCC recurrence vary from 18% to 76%[5,6] for patients who underwent standard treatment, and it is considered the major cause for no remarkable improvement in survival rates. Local and regional recurrences are dependent mainly on the site of the original tumor, clinical stage, some histological characteristics and treatment approach[5]. Local recurrence is defined as the occurrence of another carcinoma less than 2cm away from the primary carcinoma[7]. It occurs even in cases submitted to a wide excision with microscopically negative surgical margins [8,9]. In theory, local recurrences may develop both from remanescent cancer cells eventually not detected in the margins after histological

evaluation, or from genetically altered cells adjacent to the resected primary tumor [7,10].

Many clinical and pathological factors have been associated with OSCC local recurrence[11-13]. Several publications have focused on defining tumor-specific molecular markers that predict patient's outcome [15-17]. Nevertheless, tumor prognostic biomarkers and clinicopathological factors are not currently used to guide treatment decisions in clinical practice. In fact, as cited above, disease stage is the chief factor used to guide treatment and prognosis. In fact, clinicopathological factors and molecular biomarkers that could identify patients at highest risk of local recurrence are yet to be defined[17] .The aim of this matched-pair study is to identify possible clinicopathological parameters and immunohistochemical markers, as Ki-67, p53, bcl-2, FAS (Fatty acid synthase), Erb-B2, β-catenin and E-cadherin associated with early local recurrence (ELR) in patients with OSCC.

MATERIAL AND METHODS

This study was performed in 27 patients diagnosed and treated between 1981 and 2000 at the Department of Head and Neck Surgery and Otorhinolaryngology, A.C. Camargo Cancer Hospital, São Paulo, Brazil, that presented early local recurrence of OSCC. The local recurrence developed within 2cm of the treated area and ranged from 1.6 to 7.3 months after the first treatment, with a mean of 4.9 months. Subsites of oral cavity included tongue, retromolar

area, floor of mouth and lower alveolar ridge. As control, two patients with non-recurrent disease were matched to each case of the studied group according to the clinical stage, primary site location and year of admission. The inclusion criteria for all cases were biopsy proven OSCC, no prior oncologic therapy of the primary tumor and surgery as initial treatment at A.C. Camargo Hospital. The exclusion criteria were positive or close (< 5mm) surgical margins and less than 2 years of follow-up after the definitive treatment (for the control group). Clinicopathological data including age, gender, smoking and alcohol intake history, nodal status, tumor site, histological grade, perineural and lymphatic invasion, treatment, and outcome data were retrospectively obtained from the patients' medical charts. The characteristics of all patients selected for this study are summarized in Table 1. This study was carried out with approval of the Human Research Ethics Committee of the A.C. Camargo Hospital.

The paraffin embedded tissue samples were cut (3µm thickness) and mounted on silane-coated glass slides for immunohistochemistry, following standard techniques as formerly described[18]. The sections were deparaffinized, rehydrated in graded ethanol solutions. 3% hydrogen peroxide was used to block endogenous peroxidase activity. The antigen retrieval was performed by microwave in citric acid solution (pH 6.0) followed by a washing step with phosphate-buffered saline (PBS). Incubations with the following primary antibodies, diluted in PBS, were made overnight at 4º C: anti-FAS (Transduction Laboratories, Lexington, KY) 1:3000, anti-ErbB2 (Dako, Carpinteria, CA) 1:200, anti-Ki-67 MIB 1 (Dako, Carpinteria, CA) 1:100, Bcl2 (Dako, Carpinteria, CA) 1:100, E-cadherin

(Novocastra, Newcastle, UK) 1:200, β-catenin (Novocastra, Newcastle, UK) 1:200, p53 (Dako, Carpinteria, CA) 1:200. Sections were rinsed with PBS and incubated with biotinylated secondary antibodies for 30 min followed by the streptavidin-biotin-peroxidase (Strep ABC complex/HRP Duet kit, Dako) for 30 min at room temperature. Reactions were developed with a solution containing 0.6 mg/ml of 3'3- diaminobenzidine tetrahydrochloride chromogen and 0.01% hydrogen peroxide. The sections were counter stained with Carazzi's haematoxylin. Negative and positive controls were included in all reactions. Two observers (FCAA and OPA) independently evaluated and interpreted the immunostaining results without knowledge of clinical data of patients. The percentage of Ki-67 positive nuclei was calculated with the help of an image computer analyzer by counting a total of 1000 cancer cells in each sample. The median value of Ki-67–positive tumor cells was determined as 17.5% and this value separated tumors with a low or high proliferation rate. Expression of <5% of positive cells for FAS, p53, Bcl2, ErbB2, E-cadherin and β-catenin immunostaining was considered negative.

Descriptive analysis was performed to show the distribution of the population and the statistical comparisons of association between variables were performed by the Fisher's exact test or chi-square test. Kaplan-Meier method and long-rank test were used for the analysis of cumulative survival rates. The overall survival was defined as the interval between the beginning of the treatment and the date of death for uncensored observations or the last information for censored observations. Multivariate Cox's regression technique was used to build models

containing the subset of variables with independent prognostic properties. Statistical significance was determined for a p-value <0.05.

RESULTS

The early local recurrent (ELR) group consisted of 25 male and 2 female patients, with mean age of 56.4 years, ranging from 33 to 73 years, and the non-recurrent (NR) consisted of 54 patients, 45 were male and 9 female, with mean age of 55.6 years, ranging from 21 to 73 years. A significant difference in the history of alcohol consumption between the groups was observed, with values of 91.3% and 65.4% for ELR and NR, respectively (p=0.019). The primary treatment of ELR and NR patients were surgery alone in 63.0% and 38.9%, and 37.0% and 61.1% surgery with adjuvant radiotherapy, respectively. This result was statistically significant (p=0.041). Other parameters considered as age, gender, T stage, lymph node stage, clinical stage, smoking habit, histological grade, perineural and lymphatic invasion were similar for both groups (Table 1).

Immunohistochemical analysis of Ki-67, p53, Bcl2, FAS, ErbB2, E-cadherin and β-catenin protein expression is shown in Table 2 and Figure 1. For Ki-67, a threshold value of 17.5% of positive cells separated tumors with a high and low proliferation rate. Low proliferation rate was observed in 70.4% of ELR and 44.4% of NR tumors (p=0.028). p53 positivity was observed in 55.6% of ELR and 63.0% of NR tumors, while Bcl-2 cytoplasmatic expression was detected in 13.6% of ELR and 16.7% of NR cases

Two distinct patterns of ErbB2, E-cadherin and β-catenin positivity were identified. Membrane ErbB2 staining was found in 77.8% of ERL and 83.3% of NR cases, respectively, particularly in well-differentiated areas showing keratin pearls. On the other hand, cytoplasmic ErbB2 staining was found mainly in undifferentiated areas of 37.0% ELR and 42.6% NR OSCC. Membrane E-cadherin expression was found in 74.0% of ELR and 88.9% of NR tumors. Membrane β-catenin positivity was observed in 70.3% of ELR and 90.7% of NR tumors (p=0.026). FAS was expressed in the cytoplasm. The cell membrane of adipocytes stained strongly with FAS antibody and served as an additional positive control. FAS positivity was observed in 77.8% of ELR and 98.2% of NR tumors with a significant difference between the groups (p=0.005).

No statistical significant differences between ELR and NR cases were observed in the immunoexpression of p53, bcl2, ErbB2 and E-cadherin proteins (Table 2). Considering all 81 cases analyzed, some associations between biomarkers used and clinicopathological data were found (Table 3). Low proliferation rate was more observed in well-differentiated OSCC (p=0.008). Moreover, membrane ErbB2 was more expressed in OSCC of male patients (p=0.013). Cytoplasmic E-cadherin was associated with clinical and nodal stage (p=0.025 and p=0.019, respectively). Cytoplasmic β-catenin was associated with treatment performed and lymphatic invasion (p=0.028 and p=0.039, respectively). Variables associated with the 5-year and 10-year overall survival were tobacco use (0.036), alcohol consumption (p=0.014) and FAS expression (p=0.035) (Tables 4 and 5). In the multivariate analysis (Cox regression), surgery followed by

postoperative radiotherapy [Odd Ratio=0.26 (95% CI, 0.1-0.6) p=0.001] and FAS expression [Odd Ratio=0.21 (95% CI, 0.1-0.5) p=0.001] had a protective effect for ELR development.

DISCUSSION

The outcome of OSCC is often determined by clinical factors, nevertheless the clinical course of patients with similar clinical stage may differ considerably. As previously mentioned, ELR is one of the main causes for treatment failure after definitive therapy. Therefore, additional clinicopathological and molecular parameters may be valuable to understand the clinical discrepancies.

Among the demographic, lifestyle and clinicopathological features analyzed in this study, tobacco and alcohol use had a negative impact in overall survival. But, only the history of alcohol consumption was associated with ELR OSCC. It is well accepted that tobacco, particularly if associated with alcohol consumption, is the main risk factor for development of oral carcinomas [19-21]. Individual mutagen sensitivity and variability in genetically determined detoxification pathways of procarcinogenics must play a key role for the development of OSCC and patient´s outcome[20]. Our data, thus, indicate that genetic determined variations in alcohol metabolism can be involved in ELR disease development. Some studies have tried to associate alcohol dehydrogenases (ADH) genotypes with the risk for head and neck squamous cell carcinomas (HNSCC) [22-24]. ADH are enzymes that convert

ethanol to acethoaldehyde, which is a suspected oral carcinogen. Among these, the genotype ADH3 (1-2) was associated with a higher rate of HNSCC recurrence [23]. Tobacco results must be interpreted with caution on this study, since most of the patients of both groups were smokers.

Our findings clearly showed that local control was improved when surgery was associated with adjuvant radiotherapy. Combined treatment usually is performed in patients with higher risk of treatment failure, as with positive lymph nodes, vascular and perineural infiltration [4]. Local minimal residual cancer (MRC) is probably involved in ELR development in our patients, since the time of recurrence we selected was very short for nonneoplastic cells to grow into a clinically evident OSCC [7,10,25]. Patients of the NR group received more frequent adjuvant radiotherapy, and this probably helped to kill both residual cancer cells and preneoplastic cells potentially associated with field cancerization. It is interesting that despite the protective effect of postoperative radiotherapy for early treatment failure, it had no signifcant impact on patients' overall survival.

Ki-67 antibody recognizes a cell nucleus antigen, which is expressed maximally in the G2 and M phases, and it has been used widely for estimation of the growth fraction of clinical samples of human neoplasms [26,27]. The clinical value of proliferation markers for OSCC is still controversal. While some studies have shown that tumors with high proliferation rates are associated with less favorable survival and/or local control[18], other studies suggest the opposite[28]. We found lower proliferation rate related to well-differentiated tumors and in ELR OSCC [29]. Although paradoxical, this finding is in agreement with other works, showing that

low proliferation was a significant factor in local treatment failure in patients with OSCC [28,30,31] . One explanation is that OSCC with high proliferation index respond better to radiotherapy, and, in fact, this is a general rule for many tumors. Therefore, taken into consideration other factors, high proliferation index can be a signal that the tumor will respond better to adjuvant radiotherapy, possibly with less risk for local recurrence [32].

Alterations of the p53 gene are the most frequently documented genetic abnormalities in head and neck carcinomas[16,33,34]. Nevertheless, we did not find significant differences in p53 expression in ELR and NR tumors. In general, the pattern of p53 expression that we found is in accordance with other studies in patients with HNSCC [28,31]. Also, Bcl-2 positivity was similar in ELR and NR tumors and seems to play a minor role in OSCC development [35]. Overexpression of Erb-B2 has been shown in several tumors[36] such as breast[37], ovarian[38], prostate[39] and OSCC[18,40]. Several studies indicate interactions between steroid hormones receptors and Erb-B2 signaling activity, as in breast cancer, where its overexpression is inversely correlated with estrogen receptors levels[41]. Moreover, Erb-B2 increased expression was related to activate the androgen receptors signal transduction pathway and confers androgen-independent growth on prostate cancer cell line LNCaP[42]. In OSCC cell lines, Erb-B2 overexpression and decreased androgen receptors levels was reported[43]. There is a debate on the interpretation value of cytoplasmatic staining of Erb-B2[44]. Likewise other studies, our results showed that both membrane and cytoplasmic staining of Erb-B2 seem to lack correlation with recurrence development [18,40].

The E-cadherin and β-catenin complex plays a critical role in the epithelial cell-cell contact and maintenance of normal tissues[45] and the expressions of these molecules are altered in OSCC[46]. In fact, we observed that cytoplasmic E-cadherin was more expressed in advanced cases of OSCC with nodal involvement.There was a correlation between cytoplasmic β-catenin and lymphatic invasion as well as with cases treated by surgery and adjuvant radiotherapy, commonly performed in more advanced cases. Altered E-cadherin and β-catenin expressions were related to metastases since they favor cell locomotion, proteolisys, survival and proliferation in primary and distant sites[45,46]. Membrane β-catenin expression was more frequent in NR than ELR OSCC. Therefore, loss of membrane β-catenin expression should be considered as a potential marker of possible early recurrence in OSCC. We did not detect a nuclear β-catenin accumulation.

Fatty acid synthase (FAS) is a complex of seven enzymes that is required for endogenous fatty acid synthesis. Normal cells preferably utilize circulating fatty acids from dietary lipids for cellular functions and, thus, FAS is minimally expressed in most normal adult tissue[47]. However, it has been reported that tumor cells express elevated levels of FAS, and its increased expression has been associated with a poor prognosis in several human tumors [48,49]. We found that FAS expression was more common in NR than ELR OSCC. These results confirm the findings reported by other studies, in which FAS expression in OSCC was not associated with a poor prognosis[18, 50]. This seems to be the first report showing that FAS expression can be a predictive factor of treatment failure. In

addition, cases that did not express FAS protein had a significantly shorter overall survival time. These results should be confirmed by further studies.

In conclusion, according to our data, alcohol consumption was the only clinical parameter associated with early local recurrence. Postoperative radiotherapy seems to prevent ELR development in OSCC patients, and it should be considered for patients who, in one way or another, are classified as of high risk for ELR. Taken together, Ki-67, membrane β-catenin and FAS were the biomarkers associated with ELR. Therefore, patients that present OSCC with these molecular characteristics possibly should receive a more aggressive local treatment, particularly adjuvant radiotherapy or combined radiochemotherapy, along with an intensive follow up to detect eventual ELR.

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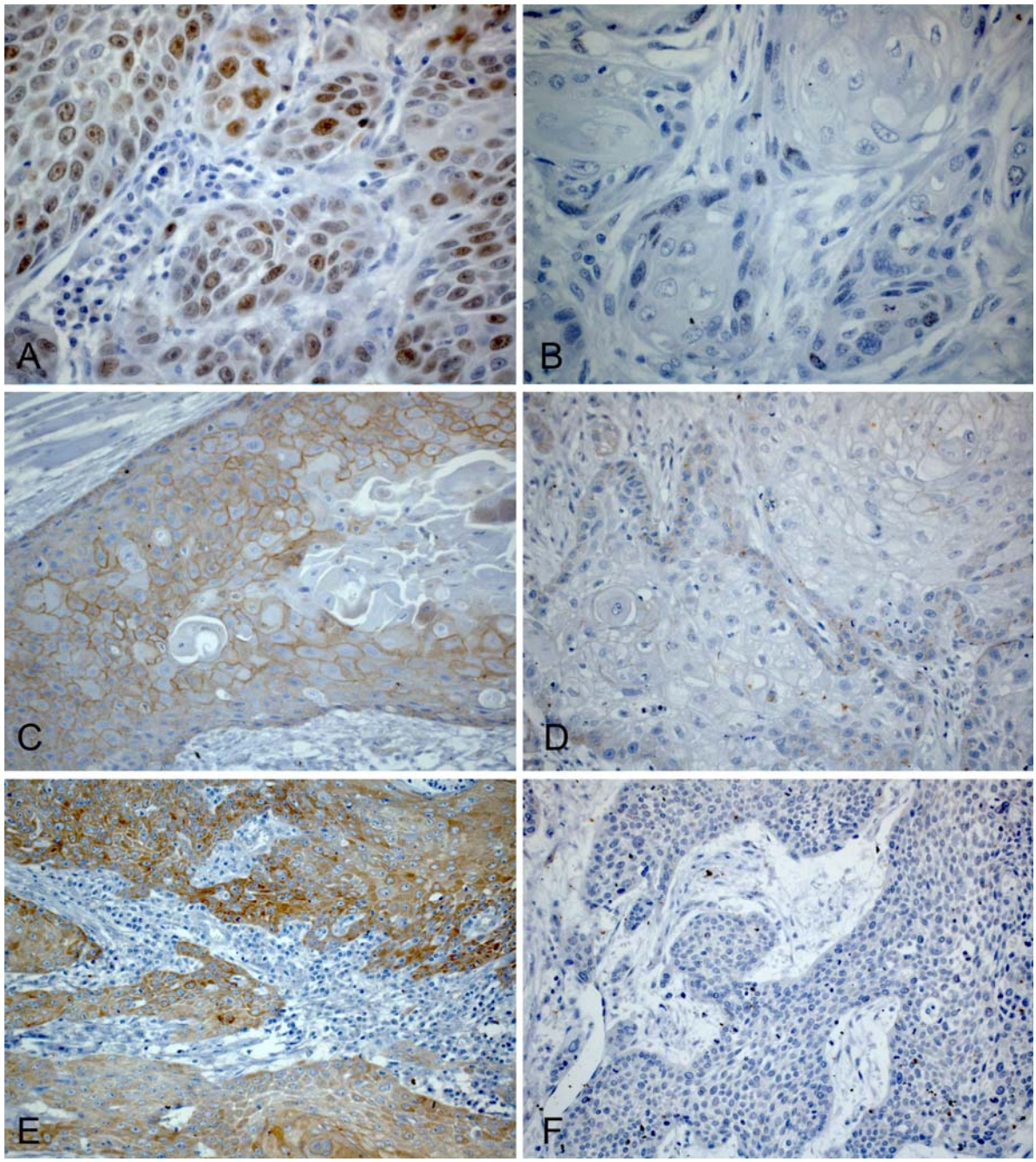

Figure 1. Immunohistochemical staining of Ki-67, β-Catenin and FAS: (A) High expression of Ki-67 in NR OSCC (original magnification 400x); (B) Low expression of KI-67 in ELR OSCC (original magnification 400X); (C and E) Membrane β-catenin and FAS expression in NR OSCC, respectively (original magnification 200x); (D and F) Loss of membrane β-catenin and no FAS immunoreactivity in ELR OSCC, respectively (original magnification 200x).

Table 1. Relationship between clinicopathological data in early local recurrent (ERL) and non-recurrent (NR) OSCC patients.

Variables	Categories	ERL n (%)	NR n (%)	p value*
Age	≤ 56	14 (51.9)	27 (50)	0.875
	≥ 56	13 (48.2)	27 (50)	
Gender	Male	25 (92.6)	45 (83.3)	0.321
	Female	2 (7.4)	9 (16.7)	
Tobacco smoking[a]	Yes	22 (95.7)	42 (80.7)	0.156
	No	1 (4.3)	10 (19.3)	
Alcohol consumption[a]	Yes	21 (91.3)	34 (65.4)	0.019
	No	2 (8.7)	18 (34.6)	
T stage	T1 + T2	8 (29.6)	20 (37.0)	0.509
	T3 + T4	19 (70.4)	34 (63.0)	
N stage	N0	13 (48.2)	28 (51.8)	0.753
	N+	14 (51.8)	26 (48.2)	
Clinical Stage	I + II	6 (22.3)	12 (22.2)	0.999
	III + IV	21 (77.8)	42 (77.8)	
Treatment	Surgery	17 (63.0)	21 (38.9)	0.041
	Surgery + RT	10 (37.0)	33 (61.1)	
Histological Grade	Well	22 (81.5)	40 (74.0)	0.458
	Moderate + Poor	5 (18.5)	14 (26.0)	
Perineural Invasion	Yes	13 (48.2)	36 (66.7)	0.108
	No	14 (43.8)	18 (33.3)	
Lymphatic Invasion	Yes	17 (63.0)	29 (53.7)	0.428
	No	10 (37.0)	25 (46.3)	

* Fisher's exact test.
[a] Excludes cases with missing information.
RT: Radiotherapy

Table 2. Relationship between immunoexpression of Ki-67, p53, bcl-2, FAS, Erb-B2, E-cadherin and β-catenin in early local recurrent (ERL) and non-recurrent (NR) OSCC.

Variables	Categories	ERL n (%)	NR n (%)	p value*
KI-67	≤17.5%	19 (70.4)	24 (44.4)	0.028
p53	+	15 (55.6)	34 (63.0)	0.520
bcl-2	+	11 (13.6)	9 (16.7)	0.321
FAS	+	21 (77.8)	53 (98.2)	0.005
Erb-B2 ^{Membrane}	+	21 (77.8)	45 (83.3)	0.557
Erb-B2 ^{Cytoplasmic}	+	10 (37.0)	23 (42.6)	0.631
E-cad ^{Membrane}	+	20 (74.0)	48 (88.9)	0.112
E-cad ^{Cytoplasmic}	+	6 (22.2)	15 (27.8)	0.789
β-Cat ^{Membrane}	+	19 (70.3)	49 (90.7)	0.026
β-Cat ^{Cytoplasmic}	+	7 (25.9)	20 (37.0)	0.317

E-cad = E-cadherin and β-Cat = β-Catenin.
* Fisher's exact test

Table 4. Cumulative overall survival in 81 patients with oral squamous cell carcinoma according to demographic, clinical and therapeutic variables.

Variables	Category	Cases	Overall Survival %		p value*
			5-years	10-years	
Age	≤ 56	41	65.7	44.2	0.820
	≥ 56	40	54.7	44.9	
Gender	Male	70	57.0	39.9	0.060
	Female	11	81.8	72.7	
Tobacco Smoking[a]	Yes	64	59.1	39.9	0.036
	No	11	81.8	71.6	
Alcohol consumption[a]	Yes	55	56.1	34.4	0.014
	No	20	79.9	69.2	
T stage	T1 + T2	28	71.3	50.4	0.256
	T3 + T4	53	54.6	41.8	
N stage	N0	41	63.2	51.3	0.194
	N+	40	57.3	36.8	
Clinical Stage	I + II	18	72.0	53.4	0.353
	III + IV	63	57.0	41.6	
Treatment	Surgery	38	57.6	44.0	0.779
	Surgery + RT	43	62.6	45.4	
Histological Grade	Well	62	54.7	39.8	0.070
	Moderate + Poor	19	79.0	61.2	
Perineural Invasion	Yes	32	65.3	50.8	0.142
	No	49	53.1	34.3	
Lymphatic Invasion	Yes	35	60.7	41.7	0.802
	No	46	59.8	48.5	

[a] Excludes cases with missing information.
* Log-rank test.
RT: Radiotherapy

Table 5. Cumulative overall survival in 81 patients with oral squamous cell carcinoma according to biomarkers.

Variables	Category	Cases	Overall Survival %		p value*
			5-years	10-years	
KI-67	≤17.5%	43	53.44	36.85	0.157
	>17.5%	38	68.06	54.12	
p53	+	49	54.79	49.07	0.532
	-	32	68.75	38.59	
Bcl-2	+	11	72.73	54.55	0.704
	-	70	58.57	43.37	
FAS	+	74	63.29	46.51	0.035*
	-	7	28.57	0.00	
Erb-B2 Membrane	+	66	63.35	45.82	0.627
	-	15	46.67	38.89	
Erb-B2 Cytoplasmic	+	33	66.54	45.15	0.621
	-	48	56.17	45.67	
E-cad Membrane	+	68	60.01	45.50	0.855
	-	13	61.54	41.03	
E-cad Cytoplasmic	+	21	60.87	49.80	0.670
	-	60	59.98	43.34	
β-Cat Membrane	+	68	64.50	48.04	0.179
	-	13	38.46	25.64	
β-Cat Cytoplasmic	+	27	70.07	58.87	0.232
	-	54	55.41	37.89	

* Log-rank test
E-cad = E-cadherin and β-Cat = β-Catenin.

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4. CAPÍTULO 2

EVALUATION OF TUMOR MARKERS IN ORAL SQUAMOUS CELLS CARCINOMAS AND IN ITS CORRESPONDING EARLY LOCAL RECURRENCES .

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SUMMARY

Early local recurrence (ELR) is one of the main causes of treatment failure in patients with oral cell squamous carcinoma (OSCC) and has a significant negative impact on prognosis. There are few studies considering the biological characteristics of the early local recurrent OSCC, particularly in relation to the primary tumor. In this study, we compared the immunoexpression of Ki-67, p53, FAS (Fatty acid syntase), Erb-B2, β-catenin and E-cadherin and the proliferation marker Ki-67 in 17 primary tumors (PT) and their respective early local recurrence (ELR). The kappa index was used for statistical analysis. p53, cytoplasmic Erb-B2, and both membrane E-cadherin and β-catenin, were expressed in primary as well as in early aggressive recurrent tumor. Ki-67, membrane Erb-B2 and both cytoplasmic E-cadherin and β-catenin showed significant changes in their expression. These differences could explain this aggressive behavior and, thus, ought to be considered in the clinical management of ELR.

KEYWORDS: Immunohistochemistry, tumor markers, local recurrence, oral squamous cell carcinoma.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is the final result of a multi-step process involving aberrant genetic events. In fact, multiple oncogenes, regulatory factors, and tumor suppressor genes play an important role in its development and progression (Scully *et al*, 2000; Vora *et al*, 2003).

In clinical practice, treatment planning and prognosis for patients with OSCC are mainly based on the tumor site, TNM classification and some histopathological criteria (Kowalski *et al*, 1993; Carvalho, Magrin & Kowalski, 2003). However, the clinical outcome cannot always be established using those parameters, since they do not provide any information regarding the biological characteristics of the tumor.

Local recurrence is defined as the occurrence of another carcinoma < 2cm away from the primary carcinoma (Braakhuis *et al*, 2002). It occurs even in cases submitted to a wide excision with microscopically negative surgical margins (Kowaslki *et al*, 1993; Brandwein-Gensler *et al*, 2005). In theory, it may develop both from cancer cells remained in the patient, which were not detected in the histological evaluation of the resection margins or from genetically altered cells adjacent to the resected primary tumor (Braakhuis *et al*, 2002; Braakhuis *et al*, 2004). Early local recurrence (ELR) is a cause for treatment failure and has a significant negative impact on the prognosis of patients of OSCC.

Evaluations of potentially useful biomarkers are performed mainly in the primary tumors, including their relevance in relation to the risk of local recurrence (Teixeira *et al*, 1996; Ginos *et al*, 2004). Nevertheless, only a few studies describe

the expression of proteins in the local recurrence itself. The aim of this study was to compare the immunohistochemical expression of biomarkers: Ki-67, p53, bcl-2, FAS (Fatty acid synthase), Erb-B2, β-catenin and E-cadherin, in OSCC primary tumors (PT) and their respective early local recurrences (ERL).

MATERIAL AND METHODS

This study included 17 patients diagnosed and treated between 1984 and 2000 at the Department of Head and Neck Surgery and Otorhinolaryngology, A.C. Camargo Cancer Hospital, São Paulo, Brazil. All of them presented early local recurrence of the disease. The inclusion criteria considered that all patients had biopsy proven OSCC, did not receive prior therapy of the PT, and were submitted to surgical treatment at A.C. Camargo Hospital. The exclusion criteria were positive margin (<5mm) after surgical treatment. Clinical data, including age, gender, smoking and alcohol intake history, TNM classification and location, treatment, and outcome data were obtained from patients' medical charts. The characteristics of patients included on this study are summarized in Table 1. This study was carried out with approval of the Human Research Ethics Committee of the A.C. Camargo Hospital. The respective paraffin-embedded block of the primary tumor and its local recurrence was taken from the pathological archives for analysis.

The paraffin embedded tissue samples were cut (3µm thickness) and mounted on silane-coated glass slides for immunohistochemistry. Immunohistochemic

staining procedure was performed by standard method as formerly described (Silva *et al*, 2004). The sections were deparaffinized and rehydrated in graded ethanol solutions. 3% hydrogen peroxide was used to block endogenous peroxidase activity. The antigen retrieval was performed by microwave in citric acid solution (pH 6.0) followed by a washing step with phosphate-buffered saline (PBS). The incubations with the primary antibodies diluted in PBS were made overnight at 4º C: anti-FAS (Transduction Laboratories, Lexington, KY) 1:3000, anti-ErbB2 (Dako, Carpinteria, CA) 1:200, anti-Ki-67 MIB 1 (Dako, Carpinteria, CA) 1:100, E-cadherin (Novocastra, Newcastle, UK) 1:200, β-catenin (Novocastra, Newcastle, UK) 1:200, p53 (Dako, Carpinteria, CA) 1:200. Sections were rinsed with PBS and incubated with biotinylated secondary antibodies for 30 min followed by the streptavidin-biotin-peroxidase (Strep ABC complex/HRP Duet kit, Dako) for 30 min at room temperature. Reactions were developed with a solution containing 0.6 mg/ml of 3'3- diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% hydrogen peroxide. The sections were counter stained with Carazzi's haematoxylin, mounted, and analyzed under an optical microscope. Positive and negative controls were included in all reactions. Two observers (FCAA and OPA) independently evaluated and interpreted the results of immunostaining without knowledge of clinical data of patients. The percentage of Ki-67 positive nuclei was calculated with the aid of an image computer analyzer (Kontron 400,Germany) by counting a total of 1000 cancer cells in each sample and assessing the percentage of labeled cells. Median value of Ki-67–positive tumor cells separated tumors with a low proliferation rate from tumors with a high proliferation rate. Expression of

<5% of positive cells for FAS, p53, Bcl2, ErbB2, E-cadherin and β-catenin immunostaining was considered negative.

The kappa index was used in the statistical analysis for the correlation of the markers expressed in the PT and its respective ELR. Values of kappa range from -1 to +1. A value <0.20 indicates weak correlation, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 good, and 0.81 to 1 excellent (Jekel *et al*, 1996).

RESULTS

The studied population consisted of 17 patients, from which only one was female. The mean age was 56.47 years, ranging from 40 to 73 years. The history of smoking and alcohol consumption was reported in 16 (94.11%) and 15 (88.23%) patients, respectively. Tumors were sited at tongue in 14 cases (82.35%) and in 3 (17.65%) at retromolar area. 5 patients were classified as clinical stages I or II, and 12 CS III or IV. 11(64.70%) patients underwent only surgical resection of the primary tumor, while 6 (35.30%) were treated with surgical resection and adjuvant radiotherapy. The time of recurrence after the first treatment was from 1.70 to 7.33 months, with a mean of 4.98 months.

Immunohistochemical expression of Ki-67, p53, FAS, ErbB2, E-cadherin and β-catenin expression in PT and ERL OSCC are summarized in the Table 2.

For statistical analysis, a threshold value of 17.5% (median) of Ki-67–positive tumor cells was used to identify tumors with a high and low proliferation index. Using this criterion, 9 PT showed low proliferation rate, and, of these, 4

(44.44%) presented high proliferation index in their respective ELR. On the other hand, of the 8 PT with a high proliferation rate, 3 (37.5%) showed a lower proliferation in their respective ELR (kappa=0.179).

All reactions with detectable p53 protein accumulation were considered positive, regardless of their intensities. FAS positivity was citoplasmatic. The cell membrane of adipocytes stained strongly with FAS antibody and served as an additional positive control. A membrane and cytoplasmic ErbB2, E-cadherin and β-catenin staining were identified and considered in our study. Membrane staining was mainly found in well-differentiated areas, such as in keratin pearls formation, whereas cytoplasmic staining was found mainly in undifferentiated cells. We did not detect nuclear β-catenin accumulation.

Membrane Erb-B2 staining was observed in 12 PT, and of these 3 (25%) were negative in their respective ELR. On the other hand, membrane Erb-B2 was undetectable in 5 PT, and was positive in 2 (40%) of its corresponding ELR (kappa = 0.337). β-catenin cytoplasmic expression was not detected in 13 PT and became positive in 2 (15,4%) of its respective ELR. E-cadherin cytoplasmic expression was not detected in 11 PT and became positive in only 1 case of ELR. Nevertheless, in those cases in which β-catenin and E-cadherin cytoplasmic staining of PT were detected, no expressions were observed in 50% of its corresponding ELR (kappa=0.326 and 0.442, respectively).

No significant changes in the immunoexpression of p53, FAS, cytoplasmic ErbB2, and both E-cadherin and β-catenin membrane were observed in PT and their respective ELR.

DISCUSSION

Accumulation of genetic alterations is the basis for OSCC development and progression, referred as multi-step carcinogenesis (Braakhuis *et al*, 2002; Braakhuis *et al*, 2004). Gene function can be altered regulating positively or negatively cell proliferation, apoptosis, genome stability, biosynthesis, angiogenesis, invasion and metastasis (Das and Nagpal, 2002). Indeed, mutations in the p53 tumor suppressor gene are the most frequently documented in head and neck carcinomas, and seem to occur early in OSCC carcinogenesis (Cordon-Cardo, 1995; Scully *et al*, 2000). Fatty acid synthase (FAS) is an enzyme required for endogenous fatty acid synthesis and is necessary for cell membrane biosynthesis (Kuhajda *et al, 1994,* Kuhajda, 2000). As reported, OSCC cell lines express elevated level of FAS and should be considered as a potential chemotherapeutic target for treatment (Agostini *et al*, 2004). Altered E-cadherin and β-catenin expressions were related to metastases, since they favor cell locomotion, proteolisys, survival and proliferation in close and distant sites (Conacci-Sorrel *et al*, 2002). Erb-B2 is a 185-KD transmembrane protein with tyrosine kinase activity and its overexpression is commonly observed in OSCC (Ulanovski *et al*, 2004). Also, the proliferating cell nuclear antigen Ki-67 has been widely used for estimation of proliferation activity (Scholzen and Gerdes, 2000). All these biomarkers seem to play an important role in OSCC behavior (Chow *et al*,

2001; Tannapfel and Weber, 2001; Das and Nagpal, 2002; Silva *et al*, 2004; Ulanovski *et al*, 2004; Thomas *et al*, 2005).

It is a well-known experience that after adequate treatment of OSCC, there is still a high risk of local recurrence. This is considered one of the main causes of treatment failure after definitive therapy (Carvalho *et al*, 2003). Cases of local recurrence are explained by the regrowth of incompletely resected tumor. However, when the histopathological assessment of surgical margins shows that the resection of the tumor was efficient, it is a clinical challenge to determine the causes that could be involved (Brandwein-Gensler *et al*, 2005). Based on clinical criterion, local recurrence is defined as the occurrence of another carcinoma < 2 cm away from the primary carcinoma (Braakhuis *et al*, 2002). One possible cause is the presence of genetic altered cells in the adjacent tissue (field), although this tissue had been considered morphologically normal by light microscope examination. When a second tumor arises from the same field in which a first tumor developed, it is commonly designated as a second field tumor (STF), since it shares the same genetic features of its related PT (Braakhuis *et al*, 2003; Braakhuis *et al*, 2005). In a study performed by van Houten *et al* (2000), in 13 head and neck carcinomas the genetic pattern of the PT, tumor-free resected margins, and local recurrences were analyzed. In 39% of the cases studied, the genetic similarity between the primary and recurrent tumor was high, providing clear evidence that residual cancer cells were the origin of recurrence. For the remaining cases a genetically related precursive lesion (field) was detected. Therefore, some cases clinically regarded as local recurrence should be, in fact, a second field

tumor, whereas local remnants of a field may develop into a new cancer (Partridge *et al*, 2000). However, molecular assays to determine the origin of local recurrence are not carried out in clinical practice (van Houten *et al*, 2000).

In our study Ki-67, membrane Erb-B2, and both cytoplasmic E-cadherin and β-catenin, showed more dissimilarity between PT and its corresponding aggressive recurrence. Those differences could be partly explained considering that some cases of ERL were STF. In spite of that, the time of disease-free interval might be considered in ELR development. Given the fact that OSCC cells need a short period of time to grow into a clinically evident tumor, not preoneoplastic cells originated from field close to resected PT, but rather local minimal residual cancer (MRC) should be involved in ELR development (van Houten *et al*, 2000).

OSCC results from a clonal evolution of abnormal cells that gains selective advantage over other cells. Within a given tumor, subpopulations of cells exist and differ in growth rates and other biologic properties (Petruzzelli, 2001). Probably, some ELR cases derived from different MRC cells from PT, and tumor heterogeneity explain the differences in biomarkers expression. Also, MRC radiosensitivity must be considered in biomarkers differences. Ionizing radiation could act causing a selection of radioresistant cells in those cases in which adjuvant radiotherapy was performed (Grabenbauer, 2000). On the other hand, p53, FAS, cytoplasmic Erb-B2, and both β-catenin and E-cadherin membrane expressions displayed no remarkable changes in PT and its corresponding aggressive local recurrence, showing a preserved biomarker expression pattern.

In summary, our results demonstrated that ELR OSCC displayed some molecular changes in its respective PT. These differences should be the key point for the aggressive recurrent behavior and these data may be useful to guide ELR treatment decision and patient's management in clinical practice.

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Table 1. Distribution of 17 cases of early recurrence of oral squamous cell carcinoma according to demographic, lifestyle and clinical variables.

Case	Age	Gender	Smoking Habit	Alcohol Use	Localization	TNM	Treatment*	Disease-free survival**
1	42	Male	Yes	Yes	Tongue	310	Sur/RT	6.2
2	51	Male	Yes	Yes	Tongue	310	Sur/RT	5.7
3	51	Male	Yes	No	Retromolar	210	Surgery	1.9
4	73	Female	Yes	Yes	Tongue	200	Surgery	5.7
5	72	Male	No	Yes	Tongue	200	Surgery	6.0
6	49	Male	Yes	Yes	Tongue	310	Surgery	5.8
7	57	Male	Yes	Yes	Tongue	400	Surgery	3.6
8	69	Male	Yes	No	Tongue	100	Surgery	4.2
9	58	Male	Yes	Yes	Tongue	310	Sur/RT	6.9
10	49	Male	Yes	Yes	Tongue	100	Surgery	1.7
11	65	Male	Yes	Yes	Tongue	100	Surgery	6.0
12	70	Male	Yes	Yes	Tongue	300	Surgery	3.8
13	60	Male	Yes	Yes	Retromolar	400	Surgery	4.8
14	40	Male	Yes	Yes	Retromolar	42a0	Sur/RT	3.6
15	45	Male	Yes	Yes	Tongue	210	Surgery	4.9
16	53	Male	Yes	Yes	Tongue	300	Sur/RT	7.3
17	56	Male	Yes	Yes	Tongue	400	Sur/RT	6.7

* Sur/RT= Surgery and postoperative radiotherapy
**Time in Months

Table 2. Imunoexpression of Ki-67, p53, FAS, Erb-B2, E-cadherin and β-catenin in the primary tumor (PT) and their respective early local recurrence (ELR).

PT	ERL		kappa
Ki-67	≤17.5%	>17.5%	
≤17.5%	5	4	0.179
>17.5%	3	5	
p53	-	+	
-	7	1	0.881
+	0	9	
FAS	-	+	
-	3	1	0.673
+	1	12	
Erb-B2 ^{Membrane}	-	+	
-	3	2	0.337
+	3	9	
Erb-B2 ^{Cytoplasmic}	-	+	
-	8	2	0.767
+	0	7	
E-cad ^{Membrane}	-	+	
-	7	2	0.767
+	0	8	
E-cad ^{Cytoplasmic}	-	+	
-	10	1	0.442
+	3	3	
β-Cat ^{Membrane}	-	+	
-	2	2	0.604
+	0	13	
β-Cat ^{Cytoplasmic}	-	+	
-	11	2	0.326
+	2	2	

E-cad = E-cadherin and β-Cat = β-Catenin.

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5. CONCLUSÕES

1 O relato do consumo de álcool, a abordagem terapêutica instituída e a imunoexpressão tumoral de Ki-67, FAS e β-catenina foram fatores que diferiram significantemente entre os pacientes portadores de carcinoma espinocelular bucal com recidiva local precoce e aqueles sem história prévia de recorrência.

2 Carcinomas espinocelulares bucais e suas respectivas recidivas locais precoces demonstraram diferenças significativas na imunoexpressão Ki-67, Erb-B2 (membrana), E-caderina e β-catenina (ambos citoplasma).

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ANEXO 1

CENTRO DE TRATAMENTO E PESQUISA
HOSPITAL DO CÂNCER
A. C. CAMARGO

São Paulo, 10 de dezembro de 2003.

Ao
Dr. Francisco Carlos A. de Aguiar Jr

Ref.: Projeto de Pesquisa n.º 556/03
"Análise clínica, histopatológica e imunohistoquímica de carcinoma espinocelular bucal em pacientes com recidiva local precoce".

Prezado Doutor:

Seu projeto de pesquisa, acima mencionado, foi apreciado pela Comissão de Ética em Pesquisa (CEP) do Hospital do Câncer em sua última reunião de 09.12.2003. Os membros desta comissão ***aprovaram*** a realização deste estudo.
Informações a respeito do andamento do referido projeto deverão ser encaminhados à secretaria do CEP dentro de 12 meses.

Atenciosamente,

Dr. Agnaldo Anelli
Presidente da Comissão de Ética em Pesquisa

C.C.
Orientador: Dr. Luiz Paulo Kowalski

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ANEXO 2

Submissions Being Processed for Author Francisco Arnanajás Aguiar, DDS MsC

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
View Submission View Artwork Quality Results		Clinicopathological and immunohistochemical evaluation of oral squamous cell carcinoma in patients with early local recurrence: a comparative study.	Feb 09 2006	Feb 09, 2006	Submitted to Journal

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ANEXO 3

Oral Diseases
Head & Neck Sciences

You are logged in

Submission Confirmation

Thank you for submitting your manuscript to *Oral Diseases*.

Manuscript ID:	ODI-02-06-OM-0331
Title:	Comparative evaluation of tumor markers in OSCC and its corresponding early recurrence.
Authors:	de Aguiar, Francisco Carlos Amanajás; University of Campinas, Oral Pathology de Almeida, Oslei Paes; University of Campinas, Oral Pathology Kowalski, Luiz Paulo; AC Camargo Hospital, Head and Neck Surgery and Otorh
Date Submitted:	09-Feb-2006

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