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FACULDADE DE ODONTOLOGIA DE PIRACICABA

ADRIELE FERREIRA GOUVÊA VASCONCELLOS
Cirurgiã-dentista
Mestre em Estomatologia

Análise clinicopatológica, da expressão imunoistoquímica de ki-67, mcm2 e geminina e da ploidia do DNA em leucoplasia verrucosa proliferativa

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutor em Estomatopatologia, área de concentração em Patologia.

Orientador: Prof. Dr. Márcio Ajudarte Lopes

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Prof. Dr. MARCIO AJUDARTE LOPES

Prof. Dr. NORBERTO NOBUO SUGAYA

Profa. Dra. LUCIELMA SALMITO PINTO

Prof. Dr. EDGARD GRANER

Prof. Dr. PABLO AGUSTIN VARGAS

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RESUMO

Leucoplasia verrucosa proliferativa (LVP) tem como principais características acometer principalmente mulheres, com idades acima dos 60 anos, sem hábitos nocivos, com lesões multifocais, recorrentes após excisão e com altas taxas de malignização. Alguns estudos com proteínas que podem estar envolvidas no controle do ciclo celular, incluindo Mcm2 e geminina, e análise de ploidia do DNA têm sido realizados com o objetivo de identificar lesões com maior predisposição para transformação maligna. **Objetivos:** Determinar a correlação das características clinicopatológicas de 21 pacientes com diagnóstico de LVP a seu estado de ploidia do DNA e expressão de Ki-67, Mcm2 e geminina. **Métodos:** 65 amostras de biópsia de 21 pacientes com LVP e 12 amostras de mucosa oral normal foram coletados, feitas imunoistoquímicas para ki-67, Mcm2 e geminina e realizada a análise da ploidia do DNA utilizando citometria de imagem (ACIS III); **Resultados:** Relação mulher: homem foi de 6:1 e a média de idade foi 65,5 anos. Dos 21 casos, 17 (80,96%) não reportaram fumo ou consumo de bebidas alcoólicas. Transformação maligna foi observada em nove pacientes (42,86%), em um tempo de seguimento clínico de 7,38 anos. Dos 21 pacientes, vinte tiveram seu DNA analisado por citometria de imagem e aneuploidia foi encontrada em 95,24% dos casos. A freqüência e severidade da aneuploidia e valores médios do índice de heterogeneidade (HI) aumentaram de acordo com o aumento das anormalidades epiteliais ($p<0.0001$), assim como as frações excedentes de 5n ($p=0.0007$). Os casos que desenvolveram carcinoma não apresentaram status aneuplóide mais grave do que as outras amostras (a maioria foi moderadamente aneuplóide). Em cinco casos as biópsias iniciais apresentando hiperqueratose e acantose ou displasia leve mostraram status aneuplóides e progrediram para carcinoma (55,5%). Não houve correlação entre os graus de displasia e a expressão de das diferentes proteínas estudadas, exceto para Mcm2 ($p= 0.0317$). **Conclusões:** Estes achados associados aos altos índices de anormalidades na ploidia do DNA podem contribuir para previsão de áreas com chances de malignização e suportam a afirmação de que LVP é uma entidade de fato distinta.

Palavras-chave: LVP, câncer oral, ploidia do DNA, imunoistoquímica.

ABSTRACT

Proliferative verrucous leukoplakia (PVL) presents as main characteristics: affects mostly women, with ages over 60 years, not presenting harmful habits; presence of multifocal lesions, recurrent after excision, with high malignization rates. Some studies with proteins that may be involved in the cell cycle control, including Mcm2 and geminin, and DNA ploidy analyzis has been performed aiming to identify lesions with a greater predisposition to malignant transformation. **Objectives:** To determine the correlation of the clinicopathological features of 21 PVL patients with their DNA ploidy status and Mcm2, geminin and Ki-67 expression. **Methods:** 65 biopsy specimens of 21 PVL patients and 12 normal oral mucosa were collected; immunohistochemistry to Mcm2, geminin and ki-67 and DNA ploidy analysis using image based citometry (ACIS III) were performed. **Results:** Female: male ratio was of 6:1 and the average age was 65.5 years. Of the 21 PVL cases, seventeen (80.96%) did not report smoking or alcoholic habit. Malignant transformation was observed in nine patients (42.86%). Of the 21 patients, twenty had the DNA examined by an image-based cytometry and aneuploidy was found in 95.24% of the cases. The frequency and severity of aneuploidy and the mean values of DNA HI increased according to the epithelial abnormality ($p<0.0001$), as well the 5n exceeding fractions ($p=0.0007$). Cases that developed carcinoma did not presented higher ploidy status compared to the other samples (the majority was moderately aneuploidy). In five cases (55.5%), initial biopsies presenting hyperkeratosis and acanthosis or mild dysplasia showed aneuploid status and latter developed carcinoma. There was no correlation between the grades of dysplasia and the LI of different immunohistochemically studied proteins, except for Mcm2 ($p= 0.0317$). **Conclusions:** This finding associated to the high incidences of DNA ploidy abnormalities may contribute to predict areas prone to malignant transformation and to support the hypothesis that PVL is a distinct entity.

Key-words: PVL, oral cancer, DNA ploidy, immunohistochemistry.

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INTRODUÇÃO

Leucoplasia verrucosa proliferativa (LVP) foi descrita em 1985 por Hansen e colaboradores, que observaram ocorrência de múltiplas lesões leucoplásicas com progressão clínica e microscópica, acometendo principalmente pacientes do gênero feminino, sem história relevante de fumo/etilismo. Em 1994 Khan et al reportaram quatro casos analisados por citometria de fluxo; em 1995 Palefsky et al reportaram mais 7 casos que apresentaram positividade para o vírus HPV 16. Em 1996, Zakrzewska et al demonstraram mais dez outros casos apresentando as mesmas características clinicopatológicas. Assim, ano após ano novos casos foram sendo relatados até que em 2005 a Organização Mundial da Saúde, em seu livro "Pathology and Genetic – Head and Neck tumors", a incluiu na seção de "Condições com potencial de malignização". Sua definição traduz o descrito em todos os estudos prévios e afirma que seu diagnóstico se baseia na associação das características clínicas e histopatológicas pregressas, que devem mostrar progressão contínua (Hansen et al., 1985; Murrah & Batsakis, 1994; Zakrzewska et al., 1996; Silverman & Gorsky, 1997; Fettig et al., 2000; Bagan et al, 2003, Bagan et al, 2004; Barnes et al, 2005; Morton et al., 2007; van der Waal & Reichart, 2008; Gouvêa et al, 2010).

LVP pode acometer qualquer local da cavidade oral podendo se iniciar como lesão única. Com o decorrer do tempo, o desenvolvimento de lesões múltiplas e multifocais é observado. Rebordos alveolares, gengiva, língua e mucosa jugal são os locais mais acometidos (Hansen et al, 1985; Batsakis et al, 1999; Fettig et al, 2000, Gouvêa et al, 2010). As taxas de malignização são altas, com estudos relatando até 100% dos casos apresentando desenvolvimento de carcinomas (Hansen et al, 1985; Zakrzewska et al, 1996; Silverman & Gorsky, 1997; Fettig et al, 2000; Bagan et al, 2003; Morton et al, 2007; Gouvêa et al, 2010). LVP apresenta alta taxa de recorrência e resistência a qualquer tratamento escolhido (Hansen et al., 1985; Murrah & Batsakis, 1994; Silverman & Gorsky, 1997; Batsakis, 1999; Lopes et al., 2000; Cabay et al., 2007).

O uso de tabaco e de álcool não tem sido diretamente associado à LVP (Eversole, 1997; Barnes et al., 2005; Cabay et al., 2007). Entretanto, vários estudos relatados na literatura mostram que pelo menos 30% dos pacientes relatam uso de fumo (prevalência média de uso de fumo - associado ou não a betel - igual a 37%) (Hansen et al., 1985; Murrah & Batsakis, 1994; Zakrzewska et al.,1996; Silverman & Gorsky, 1997; Fettig et al., 2000; Bagan et al., 2003; Morton et al., 2007). Entretanto, há aqueles que descrevem nenhum ou quase nenhum paciente fumante (Vigliante et al, 2003; Shopper et al, 2004; Gouvêa et al, 2010).

A presença de HPV em leucoplasias orais foi descrita em vários trabalhos (Miller et al., 1996; Miller & Johnstone, 2001; Campisi et al., 2004; Chen et al., 2006). Embora alguns autores tenham descrito a importância do HPV na etiologia da LVP, particularmente HPV 16 (Gopalakrishnan et al., 1997) e 18 (Palefsky et al., 1995; Femiano et al., 2001), outros autores mostram que a prevalência de HPV em LVP não difere das leucoplasias orais convencionais (Fettig et al., 2000; Eversole, 2000; Campisi et al., 2004; Bagan et al., 2007). Outro fator etiológico sugerido por Bagan et al, 2008, é o vírus EBV, entretanto, este é um dado que necessita de maiores estudos.

Os altos índices de transformação maligna apresentados por LVP fogem do comportamento esperado das leucoplasias orais, que apresentam taxas de transformação maligna abaixo dos 10% (Silverman et al, 1976; Pindborg et al, 1977; Gupta et al, 1980; Silverman et al, 1984; van der Waal et al, 1997), podendo permanecer estáveis ou até regredir em alguns casos (Pindborg et al., 1977; Gupta et al, 1980). Dessa forma, vários estudos vêm sendo conduzidos na tentativa de se elucidar o comportamento agressivo de LVP.

O estudo da expressão da proteína p53 por Gopalakrishnan et al. (1997) mostrou que houve marcação mínima em mucosa oral normal, positividade em oito de dez casos de LVP e em sete de dez casos de CEC orais. Fettig et al (2000) encontraram expressão de p53 em quatro dos dez casos analisados de LVP em gengiva e Gouvêa et al (2010) mostraram imunoexpressão em 14 dos 18 amostras selecionados. A expressão de ki-67 em leucoplasias orais é, segundo

Piatelli et al. (2003), semelhante à encontrada em p53. Assim como em estudos com p53, existem relatos na literatura afirmando que há aumento da expressão de ki-67 conforme o avanço do grau de displasia epitelial (Girod et al., 1993; Liu et al., 1998; Liu et al., 2000; Oliver et al., 2000; Kodani et al., 2001; Kövesi & Szende; 2003). De modo similar à p53, a expressão de ki-67 não foi compatível com a progressão de displasia epitelial, no trabalho de Gouvêa et al (2010).

Mcm2, Mcm5 e geminina são novos marcadores prognósticos bastante promissores estudados em trabalhos recentes. Mcm (proteínas mantenedoras de minicromossomos) é uma família composta por dez proteínas altamente conservadas em eucariotos. As proteínas Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 e Mcm7 se unem formando um complexo hexamérico que como função permitir o início da replicação do DNA celular, após ativado por diferentes fatores. Geminina é uma proteína estritamente ligada a este processo, pois regula e interage com os fatores de ativação do complexo Mcm2-7. Se há silenciamento ou inibição da ação de geminina ocorre mais de uma replicação por ciclo celular. Dessa maneira, há grandes chances de amplificação de oncogenes, aumento de instabilidades cromossômicas por acúmulo de mutações e, por conseguinte, elevação do risco de desenvolvimento de fenótipo maligno (Freeman *et al,* 1999; ; Alison *et al*, 2002; Gozales *et al*, 2005; Tachibana *et al,* 2005; Maiorano *et al,* 2006). Os poucos trabalhos que correlacionam estas proteínas a carcinomas espinocelulares de cabeça e pescoço mostram que há aumento de expressão em tumores mais agressivos, tanto clinicamente quanto microscopicamente. Torres-Rendon *et al,* em 2009, demonstraram aumento da expressão de Mcm2 e geminina conforme o aumento do grau de displasia epitelial em leucoplasias orais (Kodani *et al*, 2003; Scott *et al*, 2006; Torres-Rendon *et al*, 2009) e Gouvêa *et al* , em 2010, estudando LVP, notaram que Mcm2 e Mcm5 apresentaram maiores índices de imunopositividade e maior extensão de marcação do epitélio que ki-67.

A carcinogênese é resultado de alterações genéticas e epigenéticas, que podem levar a alterações numéricas ou estruturais cromossômicas e isto pode ser detectado pela análise da ploidia do DNA (Califano et al, 2000; Haroske et al,

2001; Kristensen et al, 2003; Fang et al, 2004; Yu et al, 2007; Torres-Rendon et al, 2009). Existem dois estudos relatados na literatura que mostram esta análise em LVP, porém apresentam amostra limitada e utilização de ferramentas não tão precisas quanto a citometria por imagem utilizando-se o ACIS III (Khan et *al*, 1994; Klanrit *et al*, 2007). Assim, há necessidade de maiores estudos a fim de se avaliar o valor preditivo deste método, correlacionando-o à expressão de proteínas envolvidas no controle do ciclo celular - ferramenta diagnóstica mais comum na prática diagnóstica.

Desta maneira, os objetivos deste estudo foram correlacionar os achados clinicopatológicos de pacientes com diagnóstico de LVP à expressão imunoistoquímica de Ki-67, Mcm2 e geminina ao status da ploidia de seus DNAs. Para, desta maneira, melhor entender esta condição e identificar possíveis marcadores prognósticos capazes de contribuir no manejo clínico dos pacientes.

CAPÍTULO 1

Leucoplasia verrucosa proliferativa: revisão da literatura

Proliferative verrucous leukoplakia: literature review of this distinct type of oral leukoplakia

Adriele Ferreira Gouvêa, DDS, MSc*
Rogério de Andrade Elias, DDS*
Jacks Jorge, DDS, PhD*
Márcio Ajudarte Lopes, DDS, PhD*

* Departamento de Diagnóstico Oral, Faculdade de Odontologia de Piracicaba, UNICAMP, São Paulo, Brasil.

Autor Correspondente:
Dr. Márcio Ajudarte Lopes, DDS, PhD
Departamento de Diagnóstico Oral,
Faculdade de Odontologia de Piracicaba-UNICAMP
Av. Limeira, 901- Areião
Caixa Postal 52, CEP: 13414-903.
Piracicaba-SP, Brasil.
Telefone: 19-21065319; FAX: 19-21065218.
E-mail: malopes@fop.unicamp.br

RESUMO

Leucoplasia verrucosa proliferativa (LVP) é uma forma agressiva de leucoplasia oral, que afeta principalmente mulheres acima de 60 anos, sem história de etilismo e tabagismo. Tem apresentação clínica multifocal, mostra recorrência e desenvolvimento freqüente de displasia epitelial e carcinoma espinocelular. O acompanhamento clínico criterioso é de suma importância para manejo adequado da LVP. Apresentamos uma revisão da literatura comparando as características epidemiológicas, etiológicas, clínicas e histopatológicas da LVP com a leucoplasia oral convencional.

ABSTRACT

Proliferative verrucous leukoplakia (PVL) is an aggressive form of oral leukoplakia that affects particularly women over 60 years of age, without tobacco and alcohol intake history. Has multifocal presentation, presents recurrences and always develops epithelial dysplasia and squamous cell carcinoma. Carefully clinical evaluation and frequent follow-up are very important in the PVL management. We present a review of the published data about this condition, comparing the epidemiological, etiological, clinical and histopathological characteristics of PVL to oral leukoplakia.

Palavras-chave: Leucoplasia, verrucosa, proliferativa, oral
Keywords: Proliferative, verrucous, leukoplakia, oral

RELEVÂNCIA CLÍNICA

Este trabalho visa informar aos cirurgiões-dentistas as principais características desta condição, alertando-os sobre seu comportamento clínico e histopatológico agressivo, possibilitando, desta maneira, o diagnóstico precoce de carcinoma e melhor prognóstico do paciente.

INTRODUÇÃO

A Organização Mundial de Saúde (OMS), em 1978, definiu leucoplasia oral como “placa ou mancha branca que não pode ser caracterizada clínica ou histologicamente como qualquer outra doença” e a incluiu no grupo de lesões com potencial de malignização[1]. LVP foi descrita, em 1985, como uma forma distinta de leucoplasia, que apresentava aparência verrucosa e exofítica, com elevados índices de transformação maligna e achados histopatológicos progressivos em biópsias seqüenciais - de hiperqueratose, diferentes graus de displasia a carcinoma verrucoso e CEC, fatos confirmados por outros autores em publicações posteriores[2,3,4]. A OMS (2005) caracteriza LVP como um subtipo raro de leucoplasia oral que possui etiologia desconhecida, acomete principalmente mulheres – proporção homens:mulheres igual a 1:4, com idade média de acometimento de 62 anos, apresenta risco de malignização e cujo diagnóstico deve ser feito através de associação das características clínicas e histopatológicas, as quais mostram evidência de displasia progressiva[5]. Diferentes trabalhos mostraram que LVP afeta múltiplos locais da mucosa oral, apresenta resistência aos tratamentos instituídos e índices de recorrência e transformação maligna expressivos [2,6,3,4,7,5]. Nosso objetivo é descrever as principais características desta apresentação distinta de leucoplasia, na tentativa de alertar os cirurgiões-dentistas sobre as altas taxas de malignização desta condição e a necessidade de acompanhamento e avaliação clínica cuidadosos.

REVISÃO DE LITERATURA

Epidemiologia - Quase todos os estudos realizados em pacientes portadores de LVP mostram que essa condição acomete principalmente mulheres, numa proporção homem:mulher igual a 1:4 e idades de diagnóstico variando entre 62 e 80 anos, dados corroborados pela OMS em 2005[2,8,3,9,10,5] (Tabela 1).

A incidência e a prevalência de leucoplasia oral convencional variam de acordo com a população estudada e com seus hábitos. No entanto, a maioria dos

estudos mostra prevalência que varia entre 1 e 5%, e maior ocorrência após os 30 anos de idade, havendo aumento da prevalência com o aumento da idade, especialmente para o gênero masculino[8].

Etiologia: Fumo e Betel - LVP, segundo alguns autores, não tem associação com uso de tabaco[5,11]. Entretanto, quase todos os estudos encontrados na literatura relatam que uma pequena parte dos pacientes fazia uso de fumo - associado ou não a betel (Tabela 01)[2,8,3,9,10,12]. Diferentemente de LVP, as leucoplasias convencionais têm forte associação com o tabaco. Este fato foi demonstrado em trabalho de Chen *et al.*, em que a prevalência de leucoplasias foi observada em 84,4% dos pacientes que faziam uso de tabaco[13]. Além disso, já foi demonstrado que há relação entre quantidade de cigarros consumidos por dia e risco de aparecimento de leucoplasias e existem relatos de remissão das leucoplasias orais após descontinuação do hábito tabagista[14].

Betel, combinação de sementes de areca, folha de betel, hidróxido de cálcio e tabaco, é outro fator etiológico de leucoplasia oral. O hábito de mascar betel é comum em populações de Taiwan, Índia, Papua Nova Guiné, África do Sul e países do sudeste asiático. Chen *et al.* encontraram incidência de leucoplasia oral em 69,7% das pessoas que mascavam betel[13].

Bebidas alcoólicas – Novamente vale ressaltar que LVP é condição que acomete prevalentemente mulheres de meia idade sem história de ingestão de álcool[5].

Embora o consumo de tabaco em suas variadas formas seja o principal agente etiológico das leucoplasias orais convencionais, consumo de bebidas alcoólicas também parece ter um papel importante neste processo. Sugere- se que o efeito sinergístico entre consumo de álcool e tabaco seja o pivô do desenvolvimento de leucoplasias orais convencionais[15,16]. Entretanto, Hashibe *et al.* afirmaram que há associação entre consumo de álcool e leucoplasias orais em pacientes não fumantes e não usuários de qualquer forma de tabaco[17]. O desenvolvimento de lesões com potencial de malignização depende da quantidade e da composição da bebida ingerida. Os destilados, como maior exemplo,

possuem em suas composições componentes carcinogênicos que podem ter efeito direto sobre as células da mucosa, causando dano ao DNA[17,18].

***Candida albicans*, Papilomavirus Humano (HPV) e Epstein – Barr vírus (EBV) -** Não existem estudos que associem o desenvolvimento de LVP com infecção por *Candida albicans*. Com relação às leucoplasias orais convencionais ainda fica em aberto a questão se *Candida* de fato causa displasia epitelial ou se apenas infecta tecido já alterado. Barrett *et al.* observaram associação significativa entre infecção fúngica e leucoplasias com displasia moderada e grave[19]. Além disso, notaram que lesões displásicas infectadas apresentavam tendência três vezes maior de desenvolver displasia epitelial mais grave.

HPV é uma família com mais de 120 subtipos de vírus DNA, medindo cerca de 5nm, sem envelope, icosaédricos, epiteliotrópicos que são transmitidos precocemente na vida. Dividem-se em HPV de baixo risco (6,11,1-5, 7-10, 12-15, 17, 19-30, 32, 34, 36-44, 46-51, 53-57 e 59), envolvidos na patogênese de várias lesões epiteliais, a maioria lesões hiperplásicas; e alto risco (16, 18, 31, 33, 35, 39, 45, 52), com potencial carcinogênico. Apesar de HPV ser encontrado em epitélio normal, a probabilidade de se detectar HPV em lesões com potencial de malignização é 2-3 vezes maior e em carcinomas espinocelulares é 4-5 vezes maior[20]. HPV 16 e 18 estão fortemente relacionados a carcinomas de cérvix uterino e anogenitais e, com relação aos CEC orais, alguns estudos sugerem que estes vírus podem ter papel etiológico[21]. A presença de HPV em leucoplasias orais convencionais foi descrita em alguns trabalhos[20,13] e, embora determinados autores tenham descrito a importância do HPV na etiologia da LVP, particularmente HPV 16 e 18[22,3,23] , outros mostraram que a prevalência de HPV em LVP não difere das leucoplasias orais convencionais[9,24].

Presença de EBV, vírus relacionado ao desenvolvimento de leucoplasia pilosa oral e a algumas neoplasias, como carcinoma nasofaríngeo, foi descrito por Bagan *et al.* em 6 de seus 10 casos de LVP e em 2 dos 5 casos de CEC analisados[25]. Entretanto, ainda não se sabe se EBV realmente tem papel efetivo na etiologia das LVP e dos CEC. Além disso, mesmo com a utilização de

Polymerase chain reaction – ferramenta sensível – não é possível afirmar se os vírus detectados infectavam apenas os queratinócitos ou os linfócitos do infiltrado inflamatório adjacente[25].

Nutrição - Não existe, ainda, nenhum estudo que mostre se nutrição deficiente tem ou não papel na etiologia de LVP. Segundo alguns autores, deficiências nutricionais podem estar envolvidas na patogenia da leucoplasia oral convencional. Em um estudo feito na Índia, os níveis séricos de vitamina A, B12, C, β-caroteno e folato estavam diminuídos significantemente em pacientes que apresentavam leucoplasia oral, mesmo nos não fumantes[26]. Assim, sugere-se que a ingestão de uma dieta rica em frutas e vegetais frescos pode ter um papel protetor na prevenção de câncer oral[27].

Aspectos Clínicos - LVP apresenta como características mais marcantes as alterações clínicas progressivas - com aumento do número das lesões e mudanças na apresentação clínica, recorrência após qualquer tratamento instituído e, principalmente, desenvolvimento de carcinoma em grande número de pacientes. Além disso, seu diagnóstico só poder ser realizado retrospectivamente, através da associação das características clínicas e histopatológicas[2,8,5]. Esta condição pode ocorrer em qualquer local da cavidade oral, no entanto, língua e mucosa jugal, palato duro, rebordo alveolar e gengiva são os locais mais acometidos[2,4].

Leucoplasias orais convencionais podem ocorrer como alterações isoladas, únicas, ou como lesões difusas, às vezes múltiplas. Os locais de distribuição das lesões mostram variações de acordo com o gênero e, principalmente, com as diferenças nos hábitos de cada população. São divididas, basicamente, em homogêneas e não homogêneas, havendo subdivisões. As leucoplasias orais homogêneas são definidas como tendo consistência e cor sempre constantes[27]. Leucoplasias não-homogêneas são assim denominadas por apresentar variação na cor (podendo ter áreas brancas e eritroplásicas, sendo, então, chamadas leucoeritroplasias) ou por mostrar aparência irregular, nodular ou

exofítica, com ou sem fissuras, geralmente apresentando textura firme à palpação (Figura 1 A, B, C, D)[8,27].

Aspectos Histopatológicos - Os aspectos histopatológicos principais da leucoplasia oral convencional são acantose e hiperqueratose. Displasia epitelial pode estar presente, variando de leve a grave. Em alguns casos, carcinoma *in situ* e até CEC invasivo podem ser encontrados microscopicamente[27,5].

LVP pode exibir características histopatológicas diferentes em uma mesma biópsia, em biópsias múltiplas realizadas no mesmo tempo cirúrgico, e em biópsias feitas ao longo do acompanhamento clínico[11]. Com o tempo, há progressivo aumento da queratinização, da verrucosidade superficial, da acantose, hiperplasia da camada basal, desenvolvimento de displasia epitelial e, não havendo tratamento, progressão inexorável para carcinoma verrucoso ou espinocelular (Figura 2 A, B, C)[2,8,3,9,7,10,28,11,29].

Diagnósticos Diferenciais - Um diagnóstico preciso de lesão branca oral deve ser feito através da análise de fatores etiológicos, exame clínico e exame histopatológico, se necessário, visto que existem diversas lesões orais que podem ser definidas como brancas[27] (Tabela 02).

Se após exame cuidadoso não houver diagnóstico conclusivo, biópsia é mandatória[27]. Como algumas lesões são muito extensas e/ou múltiplas, podendo apresentar diferentes aspectos ao longo de sua superfície, torna-se necessário realização de múltiplas biópsias, dando prioridade às áreas não-homogêneas. Também se deve atentar para a profundidade da biópsia, objetivando incluir na amostra tecido conjuntivo suficiente, possibilitando avaliação completa da área alterada[27]. LVP é diagnosticada pelos aspectos clínicos (lesões múltiplas, podendo apresentar variadas características morfológicas (hiperqueratose homogênea, superfície nodular, verrucosa e até áreas eritroplásicas ou ulceradas), histopatológicos (progressão para displasia e carcinoma em biópsias seqüenciais) e história clínica pregressa (maioria dos pacientes mulheres, sem história de consumo de álcool e fumo, lesões apresentando recorrência e resistência a qualquer tratamento instituído)[2,3,4,7,5,11,29].

Transformação Maligna - A maioria dos estudos descreve que as porcentagens de transformação maligna das leucoplasias orais convencionais geralmente estão abaixo de 10%[27]. Algumas características têm sido descritas na literatura como associadas a maior risco de transformação maligna das leucoplasias orais: gênero (mulheres parecem apresentar maior risco), tempo longo de duração da lesão, leucoplasia em não fumantes (leucoplasia idiopática), localização em assoalho bucal e/ou língua, morfologia não homogênea, presença de displasia epitelial e, fato mais recentemente estudado, expressão alterada de certas proteínas pelas células epiteliais[9,11,28]. De todas as características listadas, a mais importante, por hora, é presença de displasia epitelial e é de consenso comum que lesões displásicas apresentam risco bem mais aumentado de desenvolver carcinoma que lesões não displásicas[27].

LVP apresenta altas taxas de transformação maligna, como mostram os trabalhos encontrados na literatura. Fettig *et al.* relataram 60% de desenvolvimento de carcinoma, Bagan *et al.* relataram transformação carcinomatosa em 63% de seus pacientes, Silverman & Gorsky relataram 70%, Hansen *et al.* descreveram 87%, Zakrzewska *et al.* e Morton *et al.* relataram 100% de malignização em sua amostra[9, 10, 3, 2, 8, 12].

Tratamento - O manejo de lesões leucoplásicas orais convencionais deve primeiramente ser direcionado à eliminação de possíveis fatores causais, afastando a possibilidade de existência de outras lesões que não leucoplasia. Havendo persistência de alteração na ausência de possíveis fatores causais, biópsia deve ser realizada para exclusão histopatológica de outras lesões e para avaliação de presença de displasia epitelial, carcinoma *in situ* ou carcinoma[27]. A reavaliação periódica deve ser feita e, havendo suspeita de desenvolvimento de novas alterações epiteliais, recomenda-se realização de outras biópsias para confirmação do diagnóstico e decisão de possível tratamento. Algumas lesões leucoplásicas localizam-se em locais de difícil acesso, acometem grande parte da mucosa oral, ou ainda, recorrem depois de repetidas excisões cirúrgicas. Infelizmente, a maioria das lesões tratadas através de meios alternativos, como

vitaminas A e E, bleomicina e alfa-tocoferol, apresenta recorrência quando o tratamento é descontinuado e não há plena certeza de que o risco de malignização é completamente eliminado com nenhum dos tratamentos acima citados[30]. Dessa maneira, o acompanhamento dos pacientes que apresentam lesão tem que ser continuado por longo prazo, imprescindivelmente. O tratamento utilizado em LVP usualmente é a excisão cirúrgica, mas freqüentemente há recorrência da lesão no mesmo local, logo após remoção[2,8,7,28,12]. Assim, enquanto outras terapias mais efetivas não são propostas, acompanhamento clínico cuidadoso e frequente além de tratamento agressivo precoce são recomendados[11].

DISCUSSÃO

Existem algumas características que predizem maiores chances de transformação maligna das leucoplasias orais convencionais: acometimento de mulheres, não existência de fatores de risco – como utilização de tabaco e ingestão de bebidas alcoólicas, presença da lesão há longo tempo, aspecto não homogêneo e acometimento de assoalho bucal e/ou língua[27,31]. Algumas destas características também podem estar presentes nas LVP, o que talvez pudesse nos levar a considerá-la apenas uma forma muito agressiva de leucoplasia oral convencional. Entretanto, existem alguns pontos que são intrigantes e que não deixam muitas dúvidas de que LVP realmente é uma forma distinta de leucoplasia: a existência de taxas altas de transformação maligna – alguns trabalhos relatam que 100% de seus pacientes desenvolveram carcinoma verrucoso e/ou CEC[8, 12], relatos de acometimento de locais geralmente não associados a maior risco de malignização – como rebordos alveolares e palato duro, acometimento em sua maioria de mulheres com mais de 50 anos de idade, recorrência sempre presente e resistência a qualquer forma de tratamento instituído[2,8,3,9,7,10,28,5,11]. Por outro lado, algumas leucoplasias orais “convencionais” agressivas podem apresentar algumas destas características. Isto nos leva a ponderar se estas lesões são

realmente apenas leucoplasias mais agressivas ou se também se tratam de LVP, mas subdiagnosticadas.

O que se deve ter em mente é que LVP é condição distinta que deve ser diagnosticada pela associação retrospectiva de diversas características: ausência de fatores etiológicos, idade do paciente, além de alterações clínicas e histopatológicas progressivas[2,6,3,4,7,5,11,12]. Enquanto ainda não existem ferramentas eficazes de diagnóstico, é de bom senso estar alerta quanto à associação destas características e acompanhar estes pacientes de forma mais próxima e atenta, sempre realizando biópsias em áreas que mostrem mudanças em seu aspecto clínico. Desta maneira, o manejo adequado de lesões que apresentam displasia epitelial ou áreas de carcinoma pode ser realizado de forma precoce.

CONCLUSÃO

LVP é condição agressiva, que apresenta altas taxas de transformação maligna e cujo diagnóstico depende da associação das características clínicas e histopatológicas pregressas. Assim, podemos concluir que pacientes que apresentam características que possam ser sugestivas de LVP devem ser avaliados de forma cuidadosa e frequente tanto clínica quanto histopatologicamente

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TABELAS

Tabela 01 - Estudos publicados descrevendo casos de LVP, distribuição entre os gêneros, idades de diagnóstico e número de fumantes.

Autores	Ano	Pacientes	Gênero	Idade média de diagnóstico (anos)	Número de fumantes (%)
Hansen *et al.*	1985	30	6 H, 24 M	66	23,3
Zakrzewska *et al*	1996	10	5 H, 5 M	64	30
Silverman & Gorsky	1997	54	11 H, 43 M	62	31,5
Fettig *et al*.	2000	10	6 H, 4 M	65	50
Bagan *et al.*	2003	30	6 H, 24 M	71	60
Morton *et al.*	2007	3	1 H, 2 M	80	-
Klanrit et al.	2007	6	1H, 5 M	65.8	16,7

H: homens, M: mulheres.

Tabela 02 - Lesões orais brancas ou predominantemente brancas mais comuns e seus principais critérios diagnósticos.

Lesão	Critérios diagnósticos principais
Candisose pseudomembranosa	Aspecto clínico (pseudomembranas raspáveis, geralmente padrão simétrico)
Candidose hiperplásica	Aspecto clínico, histopatologia
Lesão friccional	Presença de irritação mecânica (ex: presença de cúspide dental pontiaguda)
Leucoplasia pilosa	Aspecto clínico (localização: bilateral lingual), presença do EBV
Lesão associada a restaurações dentárias	Aspecto clínico (relação com restauração dental)
Leucoedema	Aspecto clínico (padrão simétrico, desaparece quando mucosa é distendida)
Líquen plano, tipos reticular ou em placa	Aspecto clínico (padrão simétrico), histopatologia
Linha alba	Aspecto clínico (localização: mucosa jugal, linha de oclusão dos dentes)
Morsicatio	História de hábito de mordiscar, mastigar; aspecto clínico
Sífilis secundária	Aspecto clínico, presença do *T. pallidum*, sorologia
Lesões induzidas por tabaco	
Estomatite nicotínica	Aspecto clínico, história de fumo
Queratose por uso de tabaco	Aspecto clínico, local onde tabaco é colocado
Nevo branco esponjoso	História familiar, aspecto clínico (geralmente simétrico)
Papiloma e lesões similares	Aspecto clínico, histopatologia

Baseado em van der Waal et al[26] (1997).

FIGURAS

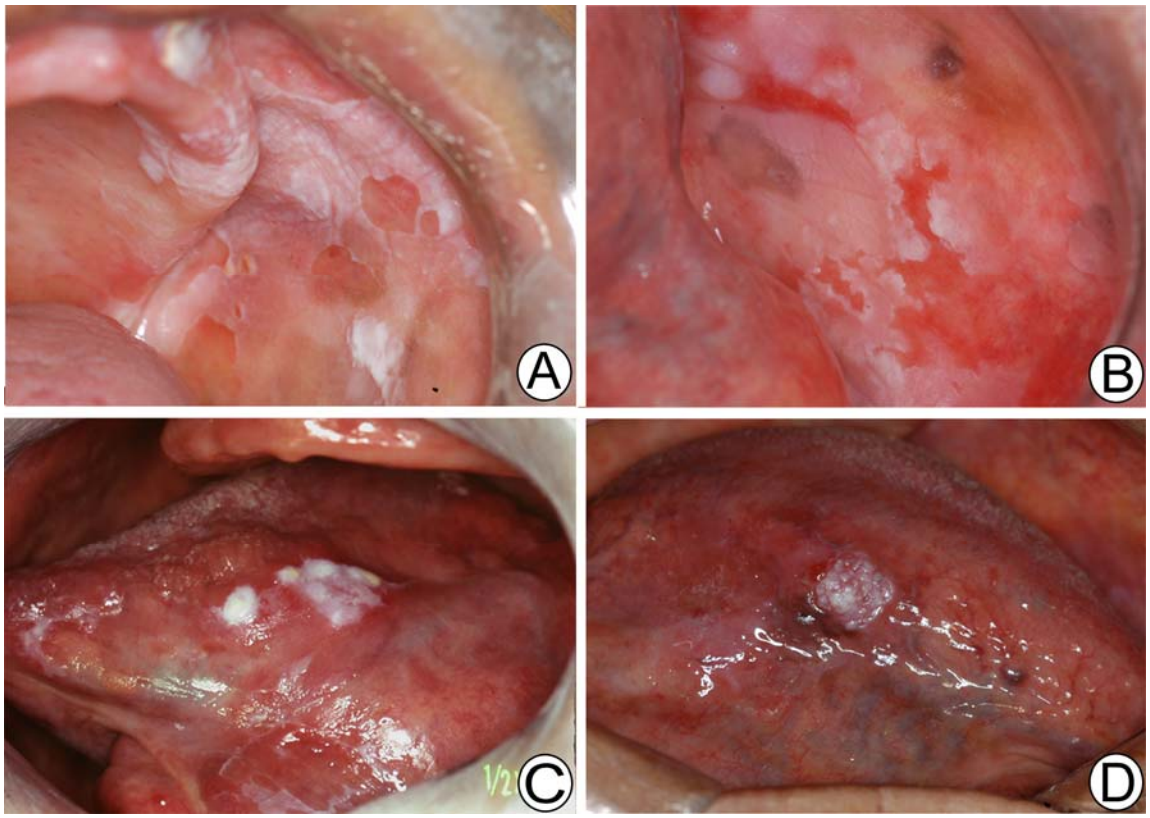

Figura 1: A- Lesões leucoplásicas em mucosa jugal, estendendo-se até rebordo alveolar superior e parte de palato duro; **B -** Área leucoeritroplásica envolvendo toda a mucosa jugal; **C -** Lesões leucoplásicas múltiplas em borda lateral de língua e assoalho bucal; **D -** Lesão leucoplásica com superfície granulosa em borda lateral de língua, clinicamente sugestiva de carcinoma.

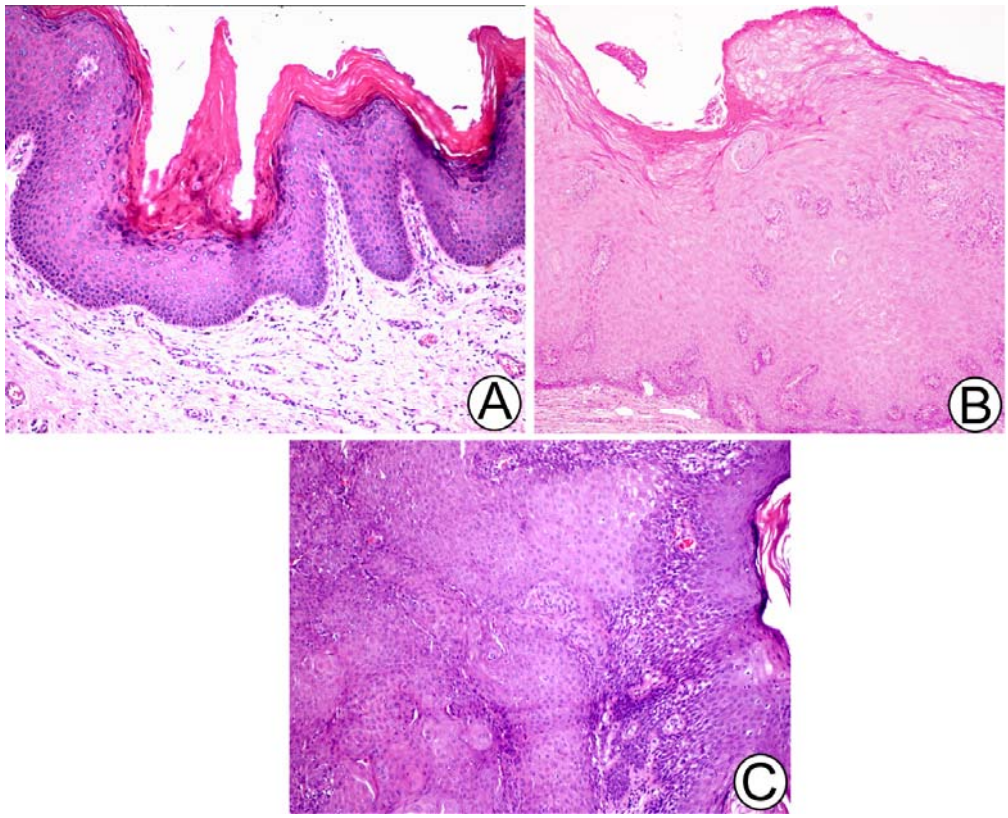

Figura 2: A – Imagem de área mostrando aumento da queratinização e verrucosidade superficial (Hematoxilina e Eosina, aumento original de 100x); **B -** Área mostrando acantose bem evidente e hiperparaqueratose (Hematoxilina e Eosina, aumento original de 100x); **C -** Imagem representativa de carcinoma espinocelular (CEC), com invasão do tecido conjuntivo (Hematoxilina e Eosina, aumento original de 100x).

CAPÍTULO 2

High incidences of DNA ploidy abnormalities associated with Mcm2 immunoexpression may contribute to predict areas prone to malignization in proliferative verrucous leukoplakia

Adriele Ferreira Gouvêa*#, Alan Roger Santos Silva*, Paul M. Speight**, Román Carlos ***, Pablo Agustin Vargas*, Oslei Paes de Almeida *, Jacks Jorge*, Márcio Ajudarte Lopes *

*Department of Oral Diagnosis, Piracicaba Dental School, State University of Campinas, São Paulo, Brazil;
** Department of Oral and Maxillofacial Pathology, The University of Sheffield, School of Clinical Dentistry, United Kingdom;
***Head and Neck Pathology Section. Centro Clínico de Cabeza y Cuello/ Hospital Herrera-Llerandi, Guatemala.

 # Corresponding author:
Dr. Adriele Gouvêa
Departamento de Diagnóstico Oral,
Faculdade de Odontologia de Piracicaba-UNICAMP
Av. Limeira, 901 - Areão
Caixa Postal 52, CEP: 13414-903
Piracicaba-SP, Brazil
Phone: +55-19-21065320 FAX: +55-19-21065218.
E-mail: adrielefg@yahoo.com.br

ABSTRACT

Proliferative verrucous leukoplakia (PVL) is a distinct form of oral leukoplakia, with unknown etiology and somewhat controversy diagnosis. Gender and age (mainly women in the 60´s decade), no evident harmful habit, multifocality, persistency and recurrence are hallmarks, besides its high rates of malignant transformation. A deregulated cell cycle may lead to numeric and structural chromosomal abnormalities, affecting the ploidy status. Many proteins may be involved in this process, including minichromosome maintenance protein 2 (Mcm2) and geminin, which are highly expressed in many malignant and premalignant conditions. It has been proven that prognosis of dysplastic lesions and tumors in early stages could be predicted by DNA content or by its aneuploid status. The aim of this study was to determine the correlation of the clinicopathological features of 21 PVL patients to the ploidy status and its Mcm2, geminin and Ki-67 expression. The female: male ratio observed was of 6:1 and the average age was 65.5 years old. Of the 21 PVL cases, seventeen (80.96%) did not report smoking and alcoholic habit. Nine patients (42.86%) developed verrucous or squamous cell carcinoma. There was no correlation between the grades of dysplasia and the immunohistochemical findings of Ki-67 and geminin, just for Mcm2 ($p= 0.0317$). Of the 21 patients, twenty had the DNA examined by an automated cellular imaging system (Dako, ACIS). Nineteen of 21 cases were aneuploidy (95.24%). The frequency and severity of aneuploidy increased according to the epithelial abnormality ($p<0.0001$), as well the mean values of DNA HI ($p<0.0001$) and the 5n exceeding fractions ($p=0.0007$). Cases that developed carcinoma did not show higher ploidy status than samples with different grades of dysplasia. In five cases, initial biopsies presenting hyperkeratosis and acanthosis or mild dysplasia showed aneuploid status and latter developed carcinoma. This finding associated to the high incidences of DNA ploidy abnormalities may contribute to predict areas prone to malignant transformation and to support the hypothesis that PVL is a distinct entity.

Key words: Proliferative verrucous leukoplakia; premalignant condition; DNA-image cytometry; aneuploidy; chromosomal abnormality; Mcm2.

INTRODUCTION

Proliferative verrucous leukoplakia (PVL) is an aggressive and distinct presentation of oral leukoplakia that was first described by Hansen *et al.*, in 1985. It affects mainly women (proportion women: men of 4:1), above 60 years of age, without history of alcohol and tobacco use. It has an unknown etiology and the diagnosis must be based on the association of the previous clinical and histopathological features. It develops on multiple sites of the oral mucosa, presents resistance to all types of treatment, has high rates of recurrence and malignant transformation (Hansen *et al.*, 1985; Murrah & Batsakis, 1994; Zakrzewska *et al.,* 1996; Silverman & Gorsky, 1997; Batsakis, 1999; Fettig *et al.*, 2000; Lopes *et al.*, 2000; Bagan *et al.*, 2003; Barnes *et al.*, 2005; Cabay *et al.*, 2007; Morton *et al.* 2007; van der Waal I & Reichart, 2008; Gandolfo *et al*, 2009; Cerero-Lapiedra et al, 2010; Gouvêa *et al*, 2010 *a* and *b*).

Taking in mind that the degree of epithelial dysplasia reflects the molecular changes and that it is a multistep process, several studies have been conducted to determine if the altered expression of some molecular markers involved in different cellular pathways - i.e. signaling pathways, transcription factors, prostaglandin factors, immortalization, chromossomic losses and gains; may be valuable indicators of clinical behavior (Kannan *et al*, 1996; Kilpi *et al*, 1996; Mao *et al*, 1996; Mutirangura *et al*, 1996; Cruz *et al*, 1998; Izzo *et al*, 1998; Jordan *et al*, 1998; Murti *et al*, 1998; Chang *et al*, 2000; Liu & Klein-Szanto, 2000; Rosin MP, 2000; Jiang *et al*, 2001; Oliver *et al*, 2001; Srinivasan *et al,* 2001; Epstein *et al*, 2003; Kodanni *et al,* 2003; Shirata *et al*, 2003; Sony *et al*, 2005; Shibata *et al*, 2005; Turatti *et al*, 2005; Fan *et al*, 2006; Kovezi *et al*, 2006; Szelachowska *et al*, 2006; Tortorici *et al*, 2008; Pitiyage *et al*, 2009; Papadimitrakopolou *et al*, 2009). Recent scientific evidence indicates that carcinogenesis is the result of genetic and epigenetic alterations gathering. This may lead to chromosomal instability –

numerical and/or structural aberrations – that might be detected by DNA aneuploidy. Abnormal DNA content is an early event in tumorigenesis and is associated to tumor progression and prognosis in several tumors (Califano *et al*, 2000; Haroske *et al*, 2001; Kristensen *et al*, 2003; Fang *et al*, 2004; Yu *et al*, 2007; Torres-Rendon *et al*, 2009). Ploidy analysis in PVL has been described in a few studies with small samples (Khan *et al*, 1994; Klanrit *et al*, 2007), thus, the predictive value of this diagnostic tool remains vague. Therefore, the purposes of this study were to analyze the clinicopathological characteristics, the immunoexpression of Mcm2, geminin and Ki-67, and the incidence of DNA aneuploidy and other related abnormalities in 21 PVL patients in order to better understand the clinical behavior and to identify of a possible prognostic marker that could improve the patients' management.

MATERIALS AND METHODS

The study design was a retrospective case control study, identifying the ploidy status in lesions of patients with PVL, comparing with control cases - normal oral mucosa (NOM). This study had the approval of the Research Ethics Committee for human studies, Piracicaba Dental School, São Paulo, Brazil, under the protocol number 006/2009, according to the National Health Council – Brazil Health Ministry for research in human subjects.

Patients and tissue selection

This study included 21 patients; all of them met the so far proposed criteria of PVL diagnosis (WHO, 2005; van der Waal & Reichert, 2008; Cerero-Lapiedra *et al*, 2010). Their clinical, demographic (age, gender and skin color) and social habits (tobacco and alcohol consumption) data were collected from the clinical charts of the Orocentro (Oral Diagnosis Clinic), Department of Oral Diagnosis, Piracicaba Dental School, Brazil and from the Centro Clínico de Cabeza y Cuello/ Hospital Herrera-Llerandi, Guatemala. Of each case were chosen samples of every biopsied oral site, prioritizing sequential biopsies - aiming to evaluate the histopathological, immunohistochemical and ploidy patterns and lesions'

progression profile. Thus, 65 biopsy specimens were collected, organized and stained with hematoxylin & eosin (H&E). Histopathological analysis searched for epithelial alterations, such as hyperkeratosis and acanthosis, epithelial dysplasia and presence of SCC or verrucous carcinoma, according to WHO latest guidelines (2005). It was analyzed blindly by two pathologists and when there were discrepancies, the slides were reanalyzed by these two examiners until reached a consensus. A group of 12 samples of NOM was used as a control reference for the ploidy analysis.

Immunohistochemistry

Immunohistochemistry was performed with a streptavidin-biotin method. Briefly, slides were washed in phosphate-buffered saline pH 7.4 (PBS) and incubated with one of the following primary monoclonal antibodies: anti-Ki-67 (clone MIB-1, DAKO, Carpinteria, CA, USA) diluted 1:100, anti-Mcm2 (clone CRCT2.1, Novocastra, Newcastle upon Tyne, UK) diluted 1:10 dilution, and Geminin (clone CRCT5.1, Novocastra, Newcastle upon Tyne, UK) diluted 1:10. After incubation with Mcm2 and geminin antibodies, slides were washed in PBS and incubated with the secondary antibody (StreptABComplex/HRP – Dako, diluted 1:20) for 30 min at 37ºC. The slides were then incubated with the streptavidin–biotin complex (StreptABComplex/HRP – Dako, diluted 1:20) for 30 min. Sections incubated with antibody against Ki-67 were incubated with the LSAB system (Labelled Streptavidin Biotin – Dako, diluted 1:100) for 30 min at 37º. Reactions were developed by incubating the sections with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) containing 0.01% H_2O_2. Control reactions performed by the omission of the primary antibodies did not show any staining.

Quantification Method

The Ki-67, Mcm2 and geminin immunohistochemical assessment (positive and negative epithelial cells and the total number of cells) were made by labeling index (LI), calculated with the help of an image computer analyser (Kontron 400, Carl Zeiss, Germany). A minimum of 200 cells were counted per case at x400

magnification under a light microscope. Representative fields in epithelial dysplasia were selected according to the most dysplastic areas and in squamous cell carcinoma (SCC) the invasive front was chosen. The entire thickness of the epithelium was taking into account and results were expressed as percentages of positively stained nuclei out of the total number of counted nuclei.

DNA Ploidy analysis (cytometric evaluation of DNA content)

Analysis of the DNA ploidy was performed at the Oral Pathology Laboratory of the University of Sheffield with the support of an image computer analyzer that uses optical density (ACIS III Automated Cellular Imaging System – DAKO, Denmark). This analysis was performed as previously described (Huang *et al*., 2005; Yu *et al*., 2007). Briefly, the paraffin-embedded tissues were sectioned in 7-μm slides and stained with Feulgen following the manufacturer's procedures (Blue Feulgen Staining Kit, ScyTek Laboratories Inc., USA). A quality control set of slides (calibration kit) provided by the manufacturer was run daily in the system to assure optimal function. The area of interest in each sample was identified microscopically on H&E sections and the corresponding area was examined on the Feulgen-stained slide. For each specimen, nuclei qualification of a minimum of 50 stromal lymphocytes and 400-targeted epithelial cells in the same tissue was obtained. To the controls cells mean integrated optical density (IOD) was assigned a DNA index of 1, which served as an internal diploid standard (2N; N meaning the number of copies of the chromosomes) and reference for DNA Index (DI) calculation of the epithelial target cells. Cell images of all cases were digitized; converted into pixels and quantified with respect to the integrated optical density value (IOD, which integrates the DNA content and morphological features of the target nuclei). Subsequently, high-fidelity DNA histograms representing selected cells in the different phases of the cell cycle were created by the system. The standards and guidelines of the European Society for Analytical Cellular Pathology (ESACAP) for DNA image cytometry were followed (Haroske *et al*, 2001).

Criteria for classification of DNA ploidy (analysis of the high-fidelity DNA histograms)

The criterion for the evaluation of the high-fidelity histograms has been previously described (Huang *et al*., 2005; Yu *et al*, 2007). High-fidelity DNA histograms represents the frequency of IOD values distribution and are plots of DI values *vs* the number of cells. When multiple peaks are present, the DI of the most prominent peak is considered. The DNA peak is determined and labelled as diploid (DI = 0.9 – 1.1), mild aneuploid (DI = 1.1 – 1.3), moderate aneuploid (DI = 1.3 – 1.8) and severe aneuploid (DI > 1.8). Samples considered severe aneuploidy (called tetraploid by some authors) presents DI between 1.8 and 2.2. The heterogeneity index (HI) was classified by the number of IOD cells clusters at 0.3 intervals in the histograms and it was displayed as different columns (or bars) in the histograms. Thirteen percent (13%) was considered as HI upper normal cutoff value, values between 13 and 20% were considered mild elevated and severe HI values grater than 20% were considered severe elevated (Pradhan et al, 2006).
Fractions of cells with DI exceeding 2.5 (5n exceeding fractions) was calculated and sub-classified as normal (<1% cells), mild (1% - 5% cells), or severe (> 5% of the cells exceeding 5N). ACIS III automatically calculated this algorithm. DNA histograms were classified in a blind fashion, without knowledge of the histological grading.

Statistical analysis

Statistical Analysis was carried out using the SAS System (SAS Institute Inc., Cary, NC, 2002). All data were treated as non-parametric. Therefore Chi-square, Likelihood Ratio Chi-Square (G2), Mantel-Haenszel chi-square were performed for the ploidy analyses. $P<0.05$ was accepted to indicate significance when comparing the groups. ANOVA-R, t-test analysis and General Linear Model of repeated measures analyses (GLM) were used to evaluate differences between Mcm2, Ki-67 and geminin LI in the abnormal epithelia and carcinoma groups. Wilcoxon test was used to evaluate differences in the Mcm2/Ki-67 and geminin/Ki-67 ratio.

RESULTS

Patients

In the PVL group, the mean age of the patients was 65.5 years (± 13.59 s.d.), varying from 45 to 84 years, and the great majority was women (85.71%). Seventeen (80.96%) did not report smoking and alcoholic habit.

All the patients presented multiple oral leukoplakic lesions and had at least two biopsied areas included in the study. The clinical follow-up of them was of 7.38 years (± 6.85 s.d.), ranging from 01 to 26 years.

Histological grading

Of the total 65 PVL biopsy samples, 12 were classified as acanthosis and hyperkeratosis, 23 as mild dysplasia, 3 as moderate dysplasia, 13 as severe epithelial dysplasia, 2 as verrucous carcinoma and 11 as squamous cell carcinoma (SCC) – 9 conventional and two exophytic type.

Immunohistochemistry

From the 65 PVL selected biopsies, high-quality immunohistochemical staining was available in 62 samples for Mcm2 and in 65 for geminin and ki-67. All samples had higher Mcm2 expression when compared to geminin and Ki-67. Hyperkeratosis and acanthosis showed similar LI values for the three analyzed markers, and SCC had the higher rates. Verrucous carcinoma showed lower LI when compared to SCC, reflecting its indolent clinical course. There was no correlation between the grades of dysplasia and the LI of different markers, except for Mcm2; which showed LI increasing according to the progressive epithelial alterations (Mcm2: GLM - P= 0.0317, Alpha = 0.05, R-Square = 0.197993, F Value 2.67; Ki-67: P= 0.78; R-Square = 0.063942; F Value = 0.5692; geminin: p= 0.5509, F value= 0.80, R-Square= 0.064879) (Table 1).

The Mcm2/Ki-67 ratio was higher than geminin/Ki-67 in all samples. There was no increase of the values according to the worsening of the epithelial abnormality (Mcm2/Ki-67: p= 0.82, geminin/Ki-67: p = 0.4928) (Table 2).

Pattern of DNA Histograms

DNA Histograms in Normal Controls and different grades of epithelial alterations

Frequency and severity of aneuploidy

Of the 65 PVL selected samples, two did not have sufficient tissue for ploidy study. In total, 75 samples were analyzed (63 PVL plus 12 NOM).

All control (NOM) was diploid. Of the PVL specimens 4/12 (33.3%) of hyperkeratosis and acanthosis cases and 3/23 (13.04%) of mild dysplasia were also diploid (Fig. 01). Diploid status was not observed in moderate, severe dysplasia and in verrucous/SC carcinoma. The majority of the hyperkeratosis and acanthosis and mild dysplasia samples were aneuploid (66.7% and 78.26%, respectively) and the frequency of the aneuploidy increased with worsening of the epithelial dysplasia ($p<0.0001$). Mean DI values of the peaks also showed interesting rates: 7/8 (87.5%) of the hyperkeratosis and acanthosis, 16/23 (88.9%) of the mild dysplasia, all the moderate, severe and SCC were moderate aneuploid (DI values varying between 1.3-1.8) (Fig. 02). The verrucous carcinoma samples showed mild aneuploidy (Fig. 03). However, even with this interesting findings, there was a slightly increase of the aneuploidy severity according to the histological abnormality. Just one SCC was severe aneuploid (tetraploid) (Fig. 04). Results are summarized in Table 3. (Chi-Square test: $p<0.0001$; Likelihood ratio and Mantel-Haenszel Chi-Square tests: $p<0.0001$; Phi Coefficient = 0.8341 and Contingency coefficient = 0.5898).

Heterogeneity of the cellular DNA content

The majority of the HI in the NOM varied from 11-13 (9/12; 75%) and three samples showed 14-20% values (3/12; 25%). Regarding PVL: almost half of hyperkeratosis and acanthosis (7/12; 58.33%) and mild dysplasia cases (11/21 – 52.38%) showed HI ≤ 13%. The remainder of these cases had mild elevated HI (14-20%), corresponding to 5 (41.67%) and 10 (47.62%), respectively. Moderate (3/3, 100%) and severe dysplasia (10/13; 76.92%), verrucous carcinoma (2/2 - 100%) and SCC (5/12; 41.66%) had HI rates varying between 14-20%. Five SCC

cases and one severe dysplasia presented HI>20%. Thus the HI increased according to the epithelial abnormality (p <0.0001). Data are summarized in the Table 4. (Mantel-Haenszel Chi-Square tests: p<0.0001; Chi Square test: p=0.0001; Likelihood ratio test p=0.0003; Phi Coefficient=0.7129 and Contingency coefficient=0.5805).

5n exceeding fraction

All the NOM had no cells with 5n exceeding fractions, as well the PVL samples presenting hyperkeratosis and acanthosis, mild and moderate epithelial dysplasia and verrucous carcinomas. Prevalence of 5n exceeding fractions did not increased substantially in severe dysplasia and SCC: 1/13 (7.69%) and 3/12 (25%) were slightly abnormal (5n fractions = 1-5%). One out of twelve SCC (8.33%) were severely abnormal (5n fractions > 5%). Thus, the groups slightly differed statistically significantly regarding >5n fractions. (Mantel-Haenszel Chi-square = 0.0007; Phi coefficient = 0.49) (Table 5).

Our sample was not large enough to statistically correlate severity of aneuploidy, DNA HI and 5n exceeding fraction. We just could detect a trend of more severe aneuploid cases to present more severe rates of HI and 5n exceeding fraction. Also, the sample was not large enough to correlate the immunohistochemical findings to the ploidy status and its details in DNA histograms.

Ploidy and clinical outcome

Seven PVL cases (33.3%) presented biopsied samples of the same site obtained in different clinical times. There was histopathological progression but aneuploid status in all, except two, since the first biopsy. Six of these 7 cases presented mild epithelial alterations as first microscopical alteration (two presented hyperkeratosis and acanthosis and four had mild dysplasia). Of these seven cases with areas progressively analyzed, five (71.43%) had SCC development. Some of them showed an unexpected abrupt microscopical change in a short follow-up time (mild dysplasia to SCC on the next biopsy – cases 3 and 14) and another had a longer elapsed clinical time to present malignant transformation (case 9).

Comparing always the same biopsied area, the nine cases that developed SCC showed aneuploid status varying from mild to moderate aneuploidy, just one were tetraploid (case 4) (Table 1).

DISCUSSION

Proliferative verrucous leukoplakia seems to be a separate entity, presenting distinct epidemiological, clinical behavior and high propensity to malignization. It is considered a rare form of oral leukoplakia, with approximate prevalence of 1 in 1000 cases in UK (Zakrzewska *et al,* 1996; Reichart & Philipsen, 2003; Klanrit *et al*, 2007). The fact that there is a little amount of PVL reported cases (188, according to Cerera-Lapiedra *et al*, 2010) may reflect clinical inattention and/or subdiagnosis. Nevertheless, it is quite important to stress that the trivialization of PVL diagnosis to any verrucous lesion presenting clinical and microscopic progression or to any case of multiple oral leukoplakic lesions without the clinicopathological correlation should be avoided (Ghazali *et al*, 2003; van der Waal & Reichart, 2008). Until the moment, there are no widely accepted diagnostic criteria – even with some suggestions and the most recent valuable proposal of Cerero-Lapiedra *et al.* (van der Waal & Reichart, 2008; Bishen & Sethi, 2009; Cerero-Lapiedra et al., 2010). The main clinical criteria that should not be ignored are: the majority of the patients are above 50 years of age, present no harmful habits, with multiple and/or extensive leukoplakic lesions in more than one anatomical site (presenting or not verrucous appearance or erythroplastic/ulcerated areas at the time of diagnosis). This lesions present clinical and microscopical changes over time and finally develop carcinoma (verrucous or squamous).

It is known that PVL diagnosis should be done based in demographic and behavioral features and in preceding clinicopathological characteristics. According to these already described guide marks our sample was in agreement with other studies: the majority of the patients were women (85.72%); the median age was 65.52 (± 13.59 s.d.); seventeen (80.96%) did not report smoking and alcoholic

habit, all the patients presented multiple recurrent lesions, with at least two biopsies performed during clinical follow-up (Table 1).

Alveolar mucosa and gingiva are the most affected sites in PVL patients, according to some authors (Zakrzewska *et al.*, 1996; Fettig *et al.,* 2000; Gandolfo *et al.*, 2009). The current data confirmed the findings reported in the literature: tongue was the most affected site (26 biopsies - 40%), gingiva and alveolar mucosa were the second most prevalent (21 samples - 32.30%). Eight (12.31%) were detected in buccal mucosa, six (9.23%) on the floor of the mouth, 3 (4.61%) on lip mucosa and 1 (1.5%) on hard palate.

In the median time of clinical follow-up (7.38 years, ± 6.85, s.d.) nine cases (42.86%) developed carcinoma and four of them more than one tumor (cases 3, 4, 15 and 20). Altogether, PVL sample showed 14 carcinomas development. Of these, the majority affected tongue (seven - 53.84%) and alveolar ridge (four - 30.76%).

The overall average PVL malignization rates are high: Klanrit *et al.*, 2007, described 50% ; Fettig *et al.*, 2000 - 60% ; Bagan *et al.*, 2003 - 63% ; Silverman & Gorsky, 1997 - 70% ; Hansen *et al.*, 1985 - 87% ; Zakrzewska *et al.,*1996 – 100%; Morton *et al.,* 2007 - 100%. The present malignization rates (42.85%) were somewhat below of the described, probably because some cases did not present a longer clinical follow-up.

Klanrit *et al*. (2007) assumed that of their six PVL analyzed cases, four had transformation predicted by epithelial dysplasia and affirmed that dysplasia detection has great value in PVL. Despite this interesting observation, it is important to note that several studies have been carried out aiming to discuss why there is a significant inter and intra -observer variability on grading oral epithelial dysplasia. Based on these findings, histopathological features are not always reliable in predicting which lesions could progress to carcinoma (Abbey *et al.*, 1995; Fisher *et al.*, 2004; Bouquot *et al.,* 2006; Kujan *et al.*, 2007; Warnakulasuryia *et al.*, 2008). In the current study, of the 65 PVL analyzed biopsies, the majority did not present tumor development heralded by a previous dysplastic state (Table 1).

Thereby, there is a need to identify objective markers capable to discern which lesions may present malignant transformation.

The pre-replications proteins importance as prognostic markers in the normal-dysplasia-carcinoma path has already been described in a series of non-oral mucosa works (Meng *et al.*, 2001; Stoeber *et al.*, 2001; Kodani *et al.*, 2001, 2003) and in a feel oral samples (Scott *et al.,* 2006; Kodani *et al.* 2001; 2003; Torres-Rendon *et al.*, 2009). In this latter study, geminin and Mcm2's LI increasing was observed according to the progression normal oral mucosa – dysplastic epithelium – SCC and a higher expression of Mcm2 than Ki-67 and geminin was noted. In a recent published study, Gouvêa and collaborators described higher expression of Mcm2 and Mcm5 than Ki-67 and high immunopositivity in some PVL cases presenting mild and moderate epithelial dysplasia (Gouvêa *et al.*, 2010 *b*). In the present study, all samples had higher Mcm2 expression when compared to geminin and Ki-67 and there was a statistical correlation between the grades of dysplasia and the Mcm2 LI (P= 0.0317). Again, some high LI rates were present in mild and moderate dysplasia – values comparable to severe dysplasia and carcinoma. The Mcm2/Ki-67 rate was higher than geminin/Ki-67 in all grades of epithelial alteration and carcinoma, maybe reflecting higher proportion of licensed G0-G1 cells. The Mcm2/Ki-67 in hyperkeratosis and acanthosis and mild dysplasia in some cases also showed results comparable to severe dysplasia and SCC, although not statistically significant. Nevertheless, none of these results could be related or could predict a more aggressive clinical behavior.

Alteration in DNA content – aneuploidy – is reported in a large number of human cancer and it has been speculated if lesions with potential of malignant transformation presenting aneuploid state could suffer malignization more frequently than lesions with normal DNA content, regardless its grade of epithelial dysplasia (Kristensen *et al.*, 2003; Fang *et al.*, 2004; Femiano & Scully, 2005; Torres-Rendon *et al.*, 2009). The DNA content can be analyzed by cytometry and some studies affirm that image cytometry is a more reliable and reproducible tool when compared to flow DNA cytometry (Huang *et al.*, 2005).

Furthermore, the high-fidelity DNA histograms obtained by ACIS, an image-based DNA-ploidy analyzer, provide quantitative information on the presence and degree of aneuploidy, cellular DNA heterogeneity, and 5n exceeding fractions – important parameters that may indicate poor prognosis (Haroske *et. al*., 1998; Kristensen *et al*., 2003; Yu *et al*., 2007).

Ploidy analyses in PVL have been done in two different studies. In 1994, Khan and collaborators analyzed by flow cytometry twenty-four samples of four cases. Just four samples were diploid and only one case showed aneuploidy with a more severe profile (DI varying from 2.4 to 2.6). These diploid samples were initial lesion that presented aneuploid profile when recurred. Klanrit *et al*., in 2007, analyzed six cases with thirty-six paraffin-embedded tissue with the Fairfield image-based ploidy analyzer and detected four cases with abnormal ploidy status prior to malignant transformation. Unfortunately, these previous studies described the results only in qualitative terms as diploid or aneuploid, thus, they missed other important histograms' details considered important predictors of clinical outcome in other human cancer (Lorenzato *et al*., 2000; Kronenwett *et al*., 2004). Moreover, it was not possible to define in all samples the DI values, aiming to establish the aneuploidy grades and the associated progression of the epithelial alterations, and whether the cases specified under the same heading were sequential biopsies or not.

In the present study all the biopsied sites were analyzed, with special attention the sequential ones as an effort to verify if there were progressive microscopical changes and, if so, which was the corresponding ploidy status. Also, we did evaluate if the cases with more aggressive clinical behavior presented a correspondent grade of ploidy abnormality. Malignant development was observed predicted by aneuploidy status in five out of the nine SCC cases (55.5%). Increasing of the DI and HI was observed according to the epithelial abnormality, as well >5n fractions; although some samples with different histopathological presentation showed similar mean DI values (moderate aneuploidy: varying from 1.3 to 1.8). This way, the DNA ploidy pattern presented accordance with the

epithelial abnormality grade. PVL, with its appropriate particularities, shows progressive histological continuum (Hansen *et al.*, 1985; Batsakis *et al.*, 1999; van der Waal &Reichart, 2008). Interestingly, 89.23% of the PVL specimens were aneuploid and even the more indolent microscopical findings had abnormal DNA content.

In conclusion, the current findings corroborate the already described clinicopathological profile of PVL. In addition, the present data suggests that PVL presents the pattern of progressive ploidy abnormality according to epithelial abnormality, as seen in other premalignant conditions. The use of more sensitive technique makes this study a more reliable reference for future works.

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DISCLOSURE/DUALITY OF INTEREST

The authors have no duality of interest to declare.

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TABLES

Table 1: Clinical findings, DNA ploidy status and labeling indexes of Ki-67, Mcm2 and geminin in PVL samples.

Patient	Biopsy year	Follow-up (years)	Biopsy site	Diagnosis	Ploidy Status	Labelling Index		
						ki-67	Mcm-2	Geminin
1	2003	7	Posterior right buccal mucosa	Acanthosis, hyperkeratosis	A	31%	20.2%	5.44%
	2003		Posterior right buccal mucosa	Hyperkeratosis	A	10.88%	43.3%	8.71%
	2003		Left gingival sulcus (tooth 46), left alveolar ridge	Mild dysplasia	A	26.92%	52.49%	4.74%
2	2000	10	Left buccal mucosa	Mild dysplasia	A	12.18%	42.07%	6.07%
	2005		Left buccal mucosa	Acanthosis, hyperkeratosis	A	14.61%	25.44%	5.02%
3	2003	7	Right lateral border of the tongue	SCC	A	33.33%	52.24%	13.78%
	2004		Anterior alveolar ridge and floor of the mouth	Mild dysplasia	A	16.74%	28.9%	25.28%
	2004		Floor of the mouth	Mild dysplasia	A	31.93%	26.64%	14.72%
	2005		Floor of the mouth	SCC	A	48.43%	65.71%	18.76%
4	2003	7	Lower alveolar ridge	SCC	A	25.79%	35.2%	18.5%
	2004		Left lower alveolar ridge	SCC	Tetraploid	34.43%	67.79%	25%
5	2003	7	Left lateral border of the tongue	Acanthosis, hyperkeratosis	Diploid	15.17%	24.11%	8.25%

	2007		Floor of the mouth	Mild dysplasia	**Diploid**	11.39%	29.41%	6.85%
6	**2005**	**5**	Right buccal mucosa	Acanthosis, hyperkeratosis	**A**	34.55%	26.91%	8.94%
	2005		Left lateral border of the tongue	Mild dysplasia	**A**	22.22%	32.98%	14.92%
7	**2006**	**4**	Left upper alveolar ridge	Mild dysplasia	**A**	20.52%	26.17%	4.11%
	2006		Right lower gingiva	Acanthosis, hyperkeratosis	**Diploid**	17.32%	27.92%	5.97%
	2006		Right upper alveolar ridge	Mild dysplasia	**A**	34.92%	30.35%	6.7%
	2006		Right upper alveolar ridge	Mild dysplasia	**A**	19.17%	18.39%	4.14%
8	**2006**	**4**	Gingiva (tooth 25)	Mild dysplasia	**A**	17.64%	23.9%	5.99%
	2006		Gingiva (tooth 15)	Acanthosis, hyperkeratosis	**Diploid**	15.1%	24.51%	5.4%
9	**1994**	**16**	Left posterior border of the tongue	Acanthosis, hyperkeratosis	**Diploid**	31.85%	15.22%	9.52%
	1997		Right posterior border of the tongue	Acanthosis, hyperkeratosis	**A**	7.25%	41.98%	8.87%
	2002		Left posterior border of the tongue	SCC	**A**	31.31%	68.8%	16.48%
	2007		Anterior gingival sulcus	Acanthosis, hyperkeratosis	**A**	21.76%	23.68%	12.51%
	2007		Left floor of the mouth	Acanthosis, hyperkeratosis	**A**	15.6%	29.79%	8.64%
	2007		Lower lip mucosa	Mild dysplasia	**A**	38.49%	26.52%	12.98%
	2009		Lower lip mucosa	Mild dysplasia	**A**	33.98%	28.26%	13.11%
10	**2009**	**1**	Right inferior alveolar ridge	Verrucous carcinoma	**A**	17.88%	8.87%	8.65%

	2009		Right posterior hard palate	Severe dysplasia	**A**	23.42%	35.6%	11.54%
11	**2004**	**6**	Anterior alveolar ridge	Severe dysplasia	**A**	28.21%	50%	9.25%
	2006		Anterior alveolar ridge	Severe dysplasia	**A**	51.96%	38.75%	22.53%
	2008		Anterior alveolar ridge	SCC	**A**	44.55%	73.21%	27.84%
12	**2004**	**6**	Right lateral border of the tongue	Hyperkeratosis	**A**	11.55%	30.15%	4.83%
	2008		Right floor of the mouth	Mild dysplasia	**A**	6.4%	ND	7.09%
	2008		Right lateral border of the tongue	Moderate dysplasia	**A**	0.55%	ND	7.5%
	2008		Right lateral border of the tongue	Severe dysplasia	**A**	43.13%	40.35%	40.35%
	2009		Right lateral border of the tongue	Severe dysplasia	**A**	50.83%	28.47%	12.54%
13	**2008**	**2**	Left upper alveolar ridge	Mild dysplasia	**Diploid**	14.23%	ND	7.89%
	2008		Left upper alveolar ridge	Mild dysplasia	**A**	18.09%	24.6%	6.07%
	2009		Left buccal mucosa	Mild dysplasia	**A**	19.88%	17.91%	9.67%
14	**2007**	**3**	Right dorsun of the tongue	Moderate dysplasia	**A**	46.56%	28.54%	11.13%
	2008		Right lateral border of the tongue	Mild dysplasia	**A**	20.71%	28.89%	12.98%
	2008		Right lateral border of the tongue	SCC	**A**	47.42%	69.81%	27.54%
15	**2009**	**1**	Upper labial mucosa	Exophitic carcinoma	**A**	55.88%	69.36%	17.37%
	2009		Hard palate	Exophitic carcinoma	**A**	51.22%	64.12%	21.93%

16	**2004**	**6**	Buccal mucosa	Mild dysplasia	**A**	26.29%	39.11%	12.17%
	2009		Left upper alveolar ridge	Mild dysplasia	**A**	23.42%	35.32%	3.78%
	2009		Left lower alveolar ridge	Severe dysplasia	**A**	33.23%	54.04%	9.38%
17	**1984**	**26**	Ventral surface of the tongue	Mild dysplasia	**Diploid**	44.87%	30.56%	13.37%
			Buccal mucosa	Mild dysplasia	**A**	31.05%	20.16%	9.64%
18	**1985**		Superior gingiva	Mild dysplasia	**ND**	33.74%	31.41%	12.66%
			Retromolar lower left mucosa	Mild dysplasia	**ND**	12.9%	34.6%	8.99%
19	**2007**	**3**	Left lateral posterior ventral surface of the tongue	Severe dysplasia	**A**	47.93%	32.24%	7.07%
	2007		Right lateral ventral surface of the tongue	Severe dysplasia	**A**	32.58%	28.1%	15.53%
20	**2005**	**5**	Dorsun of the tongue	Severe dysplasia	**A**	25.72%	44.69%	31.93%
	2006		Dorsun of the tongue	Verrucous hyperplasia - moderate dysplasia	**A**	37.64%	48.32%	23.47%
	2007		Dorsun of the tongue	SCC	**A**	64.87%	65.61%	25.8%
	2007		Dorsun of the tongue	SCC	**A**	54.48%	74.18%	35.32%
			Dorsun of the tongue	SCC	**A**			28.25%
21	**2006**	**4**	Tongue tip	Severe dysplasia	**A**	23.52%	17.29%	8.41%

	Left lateral border of the tongue	Severe dysplasia	**A**	20.65%	39.71%	14.28%
	Right lateral border of the tongue	Verrucous carcinoma	**A**	10.5%	19.8%	5.84%
	Dorsun of the tongue	Severe dysplasia	**A**	17.84%	37.77%	4.05%

Text highlighted in yellow: cases presenting the same biopsied site in different clinical follow-ups. In red: carcinoma cases. In green: the unique complete diploid case. ND: Not done. The cases presenting biopsies performed in the same year had these procedures done in different clinical times.

Table 2. Median, lower and upper quartiles of Mcm2, ki-67 and geminin LI and median with percentiles in the Mcm2/ki-67 and geminin/ki-67 ratios in different epithelial abnormalities and carcinoma (verrucous and squamous) in PVL sample.

	LI Median (25%-75%)				
PVL sample	**Mcm2**	**ki-67**	**Geminin**	**Mcm2/ki-67**	**Geminin/ki-67**
Hyperkeratosis and acanthosis	26.18 (21.94 - 29.97)	14.86 (9.07- 19.54)	7.31 (5.21 - 8.91)	1.62 (0.93 - 2.25)	0.38 (0.32 - 0.56)
Mild dysplasia	26.17 (17.91 - 29.41)	16.74 (0.34 - 2.22)	6.07 (0.13 - 9.67)	1.35 (0.87 - 1.50)	0.38 (0.30 - 0.60)
Moderate dysplasia	24.31 (0.29 - 48.32)	0.55 (0.38 - 46.56)	0.23 (0.11- 7.50)	0.95 (0.61 - 1.28)	0.62 (0.23 - 13.63)
Severe dysplasia	14.51 (0.39 - 38.26)	0.41(0.25 - 35.67)	0.24 (0.09 - 12.04)	1.22 (0.74 - 1.72)	0.62 (0.23 - 13.63)
Verrucous carcinoma	14.34 (8.87- 19.80)	9.00 (0.11- 17.88)	4.36 (0.06 - 8.65)	1.19 (0.49 - 1.88)	0.52 (0.48 - 0.55)
SCC	65.71 (0.69 - 69.81)	25.79 (0.54 - 34.43)	0.32 (0.26 - 17.49)	1.36 (1.25 - 1.64)	0.52 (0.40 - 0.65)
	P= 0.0317	NS	NS	NS	NS

NS: non significant; SCC: squamous cell carcinoma

Table 3. Severity of aneuploidy in normal oral mucosa (NOM) and in different PVL grades of epithelial alterations.

	Groups	n	Diploidy (%)	Aneuploidy (%)			
				Mild	Moderate	Severe	Total
Control	NOM	12	12 (100)	0	0	0	0
PVL	Hyperkeratosis and acanthosis	12	4 (33.3)	1 (12.5)	7 (87.5)	0	8 (66.7)
	Mild dysplasia	21	3 (13.04)	2 (11.1)	16 (88.9)	0	18 (78.26)
	Moderate dysplasia	3	0	0	3 (100)	0	3 (100)
	Severe dysplasia	13	0	0	5 (38.46)	8 (61.54)	13 (100)
	Verrucous carcinoma	2	0	2 (100)	0	0	2 (100)
	SCC	12	0	0	11 (91.67)	1 (8.33)	12 (100)
	Total	75	19 (25.33)	5 (8.93)	42 (75)	9 (16.07)	56 (74.67)

SCC: squamous cell carcinoma. Chi-Square test: $p<0.0001$; Likelihood ratio and Mantel-Haenszel Chi-Square tests: $p<0.0001$.

Table 4. Distribution of cases according to the DNA HI and epithelial alterations.

	Groups	n	≤13 (%)	14-20 (%)	>20 (%)
Control	**NOM**	12	9 (75)	3 (25)	0
PVL	**Hyperkeratosis and acanthosis**	12	7 (58.33)	5 (41.67)	0
	Mild dysplasia	21	11 (47.82)	10 (43.47)	0
	Moderate dysplasia	3	0	3 (100)	0
	Severe Dysplasia	13	2 (15.38)	10 (76.92)	1 (7.69)
	Verrucous carcinoma	2	0	2 (100)	0
	SCC	12	2 (16.66)	5 (41.66)	5 (41.66)
	Total	75	31 (41.33)	38 (50.67)	6 (8)

Mantel-Haenszel Chi-Square tests: $p<0.0001$; Chi Square test: $p=0.0001$; Likelihood ratio test $p=0.0003$.

Table 5. Distribution of cells with 5N exceeding rate in different PVL epithelial alterations grades.

Groups		5N exceeding fractions			
		n	≤1 (%)	1- 5 (%)	>5 (%)
Control	NOM	12	12 (100)	0	0
PVL	Hyperkeratosis and acanthosis	12	12 (100)	0	0
	Mild dysplasia	21	21 (100)	0	0
	Moderate dysplasia	3	3 (100)	0	0
	Severe dysplasia	13	12 (92.31)	1 (7.69)	0
	Verrucous carcinoma	2	2 (100)	0	0
	SCC	12	8 (66.67)	3 (25)	1 (8.33)
	Total	75	70 (93.33)	4 (5.33)	1 (1.33)

Mantel-Haenszel Chi-square = 0.0007.

FIGURES

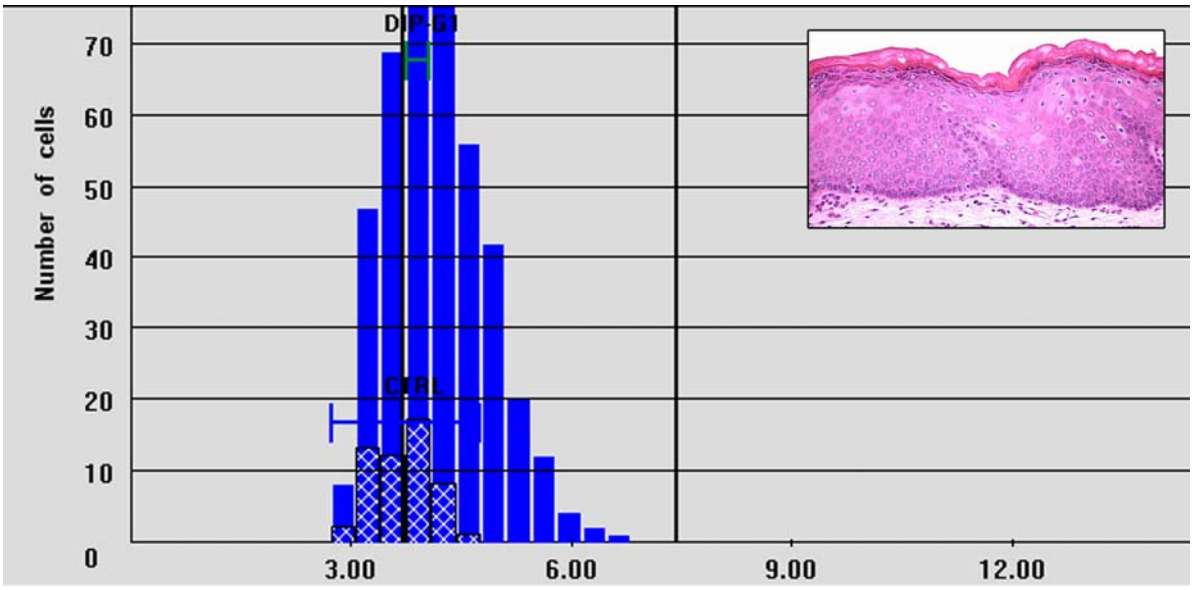

Figure 1: High-fidelity histogram of a hyperkeratosis and acanthosis case with a diploid pattern (detail – HEx200).

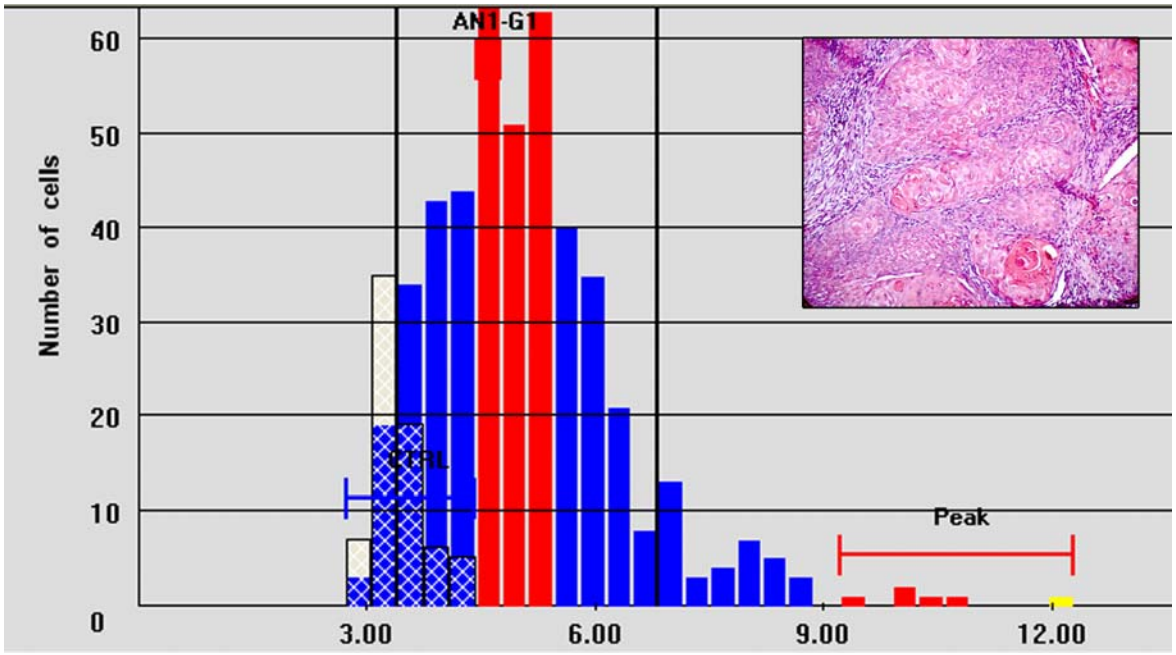

Figure 2: High-fidelity histogram of a moderate aneuploid well-differentiated carcinoma (detail – HEx100).

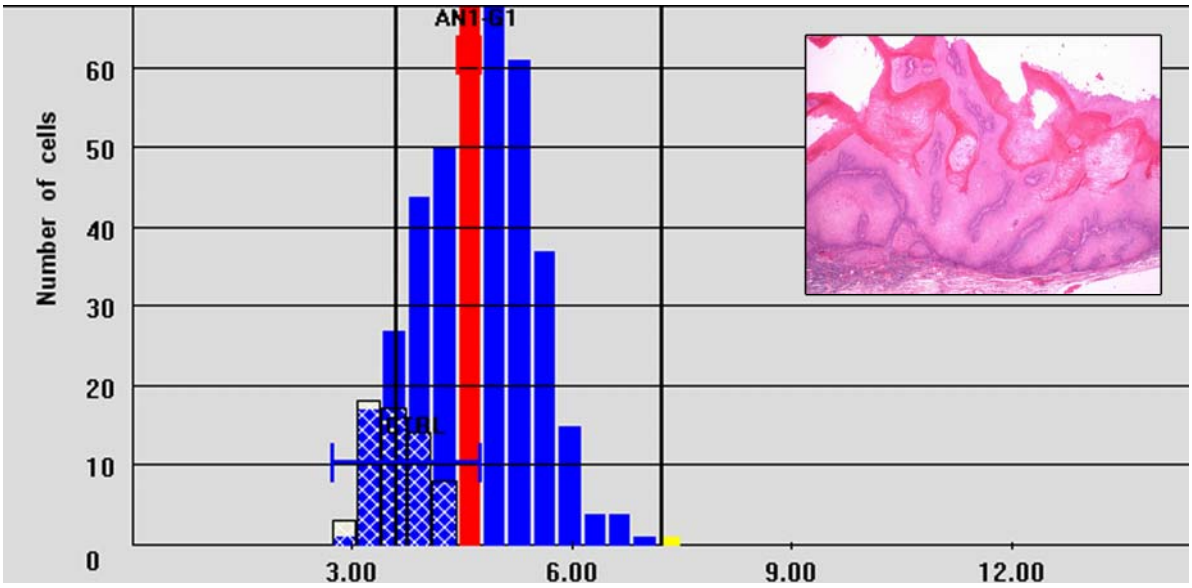

Figure 3: High-fidelity histogram of a mild aneuploid verrucous carcinoma (detail – HEx25).

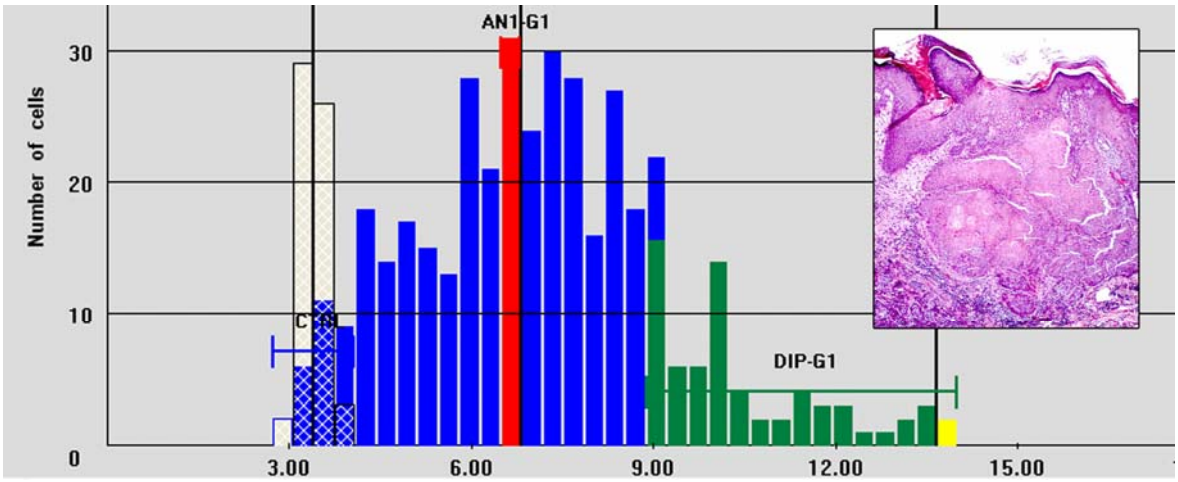

Figure 4: High-fidelity histogram of a severe aneuploid (tetraploid) undifferentiated carcinoma (detail – HEx50).

CAPÍTULO 3

LETTER TO THE EDITOR

PVL and DNA ploidy data: its current issues and highlights

Adriele Ferreira Gouvêa*, Alan Roger Santos Silva*, Paul M. Speight**, Román Carlos ***, Pablo Agustin Vargas*, Oslei Paes de Almeida *, Márcio Ajudarte Lopes *#

*Department of Oral Diagnosis, Piracicaba Dental School, State University of Campinas, São Paulo, Brazil;
** Department of Oral and Maxillofacial Pathology, The University of Sheffield, School of Clinical Dentistry, United Kingdom;
***Head and Neck Pathology Section. Centro Clínico de Cabeza y Cuello/ Hospital Herrera-Llerandi, Guatemala.

Corresponding author: Dr. Adriele Ferreira Gouvêa, DDS, MSc
Departamento de Diagnóstico Oral,
Faculdade de Odontologia de Piracicaba-UNICAMP
Av. Limeira, 901- Areião
Caixa Postal 52, CEP: 13414-903.
Piracicaba-SP, Brazil.
Phone: +55-19-21065320; FAX: +55-19-21065218.
E-mail: adrielefg@yahoo.com.br

Proliferative Verrucous Leukoplakia (PVL), first described by Hansen and collaborators in 1985, is still a somewhat controversial entity. Notoriously, the WHO and a broad quantity of studies confirm that it is a rare, distinct and high-risk clinical form of precursor lesion (Hansen et al, 1985; Murrah and Batsakis, 1994; Zakrzewska et al, 1996; Silverman and Gorsky, 1997; Fettig et al, 2000; Bagan et al, 2003; WHO, 2005; Cabay and Epstein, 2007; Bagan et al, 2010; Cerero-Lapiedra, 2010; Gouvêa et al, 2010).

There are few described cases (188, according to Cerera-Lapiedra *et al*, 2010) and the estimate prevalence in UK is approximately 1 to 1000 cases of conventional oral leukoplakia (Zakrzewska *et al,* 1996; Reichart & Philipsen, 2003; Klanrit *et al*, 2007); probably because of the underreporting or subdiagnosis (Zakrzewska et al, 1996). These facts maybe occur because there is no consensus for diagnosis, which should be based on the association of histological and clinical criteria. The main clinical criteria that should not be ignored are: the majority of the patients are above 50 years of age, present no harmful habits, with multiple and/or extensive leukoplakic lesions involving more than one anatomic site (presenting or not verrucous appearance at the time of diagnosis, or erythroplastic/ulcerated areas) which presents clinical and microscopical changes over time and finally develop carcinoma (verrucous or squamous). On the other hand, it is crucial to reinforce that the trivialization of PVL diagnosis to any verrucous lesion presenting clinical and microscopic progression or to any case of multiple oral leukoplakic lesions without the clinicopathological correlation should be avoided (Ghazali *et al*, 2003; van der Waal & Reichart, 2008).

In addition, there is no complete agreement regarding the nomenclature “PVL”. Some argue that the term “proliferative” adds confusion to the terminology (van der Waal and Reichart, 2008) – which is probably true; and some might affirm that the term “verrucous” implies that the patients must develop verrucous carcinoma or verrucous lesions. In its first PVL report, Hansen and collaborators (1985) coined this name because of the clinical characteristics of leukoplakic lesions which progressively expanding, becoming sometimes multifocal and

exophytic/verrucous/ulcerated; description later supported by Murrah and Batsakis (1994) and Zakrzewska et al (1996). Also, they described the occurrence of either verrucous *or* squamous carcinoma (Hansen et al, 1985; Silverman and Gorsky, 1997).

Not all patients will necessarily develop a verrucous lesion prior to malignant transformation. In our 21 PVL analyzed patients, it was observed that some presented development of warty and verrucous appearance (Fig. 1); while others presented lesions with clinical aspect of a more hyperkeratotic leukoplakia, similar to what was previously reported by other studies. The point is that some clinically somewhat indolent lesion presented mycroscopically severe dysplasia (Fig. 2).

Hansen et al (1985), and Zakrzewska et al (1997), stated that lesions of PVL tend to be slow-growing (up to decades), persistent and resistant to all forms of treatment. Clearly, it was described clinical and microscopical progression of the lesions – one of the PVL hallmarks. The same lesional slow-growing was observed in the majority of the present studied cases; however some of them presented a sudden aggressive progression with lesions depicting clinical changes in less than one month of follow-up (Fig. 3). A number of patients presented on the first clinical evaluation with a carcinomatous lesion and others presented several carcinomas in a short elapsed time; with one of them even developing an uncommon axillary lymph node metastasis (Gouvêa et al, 2010). It was also observed that some patients do well for years, however suddenly present important clinical changes. So, why do PVL patients present different clinical behavior? And why one patient remains stable and then suddenly presents abrupt clinical changes and malignization?

Recently our group concluded a study with PVL 21 patients trying to correlate the clinical profile to the DNA ploidy pattern, using image-based cytometry (ACIS III - based on the ESACAP criteria - Haroske et al, 2001; Huang et al, 2005). All the patients presented multiple recurrent lesions, with at least two biopsies included on the study. The majority of the patients were women (85.72%); the median age was 65.52 (± 13.59 s.d.); and seventeen (80.96%) did not report

smoking and alcoholic habit. The DNA ploidy analyzis of the biopsied samples showed interesting details. As expected, all cases demonstrated increase of the frequency and severity of aneuploidy; as well as the cellular heterogeneity and 5n fractions according to the worsening of the histological grades of dysplasia. Thereby, the DNA ploidy pattern presented accordance with the epithelial abnormality grade. About 95% of the analyzed specimens were aneuploid, and malignant development could be predicted by aneuploidy status in five out of the nine SCC cases (55.5%). In addition, and most importantly, even the more indolent microscopical findings such as hyperkeratosis and acanthosis or mild epithelial dysplasia had abnormal DNA content. On the contrary, most of the carcinoma cases presented moderate aneuploidy grade (independently of its microscopical and clinical stage). Why the clinically more aggressive cases did not present more severe aneuploid status as demonstrated by Huang et al (2007), in the classical Barrett's esophagus?

Despite these fascinating issues, this study was interesting in order to demonstrate the possible predictive DNA ploidy analyzis value. Also, it is interesting to note that some patients might present a more aggressive clinical behavior than others, therefore clinicians must be aware to the need for long-term (lifelong) and close clinical follow-up (sometimes monthly) keeping in mind that even discrete leukoplakic lesions without important dysplasia may develop malignant transformation .

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FIGURES

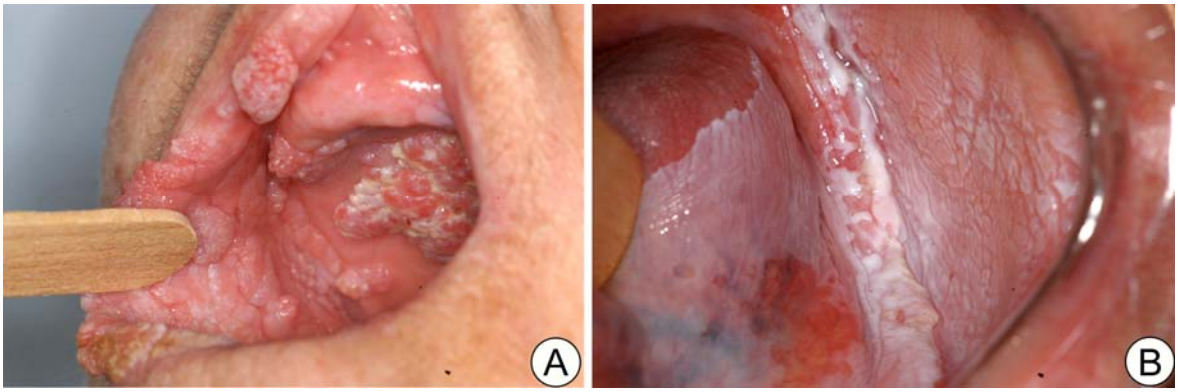

Figure 1: Two female patients showing lesions with a hyperkeratotic and verrucous surface. **A –** An exophytic carcinoma was proven on hard palate and alveolar mucosa biopsies. **B –** A micro-invasive carcinoma was detected in this posterior alveolar ridge.

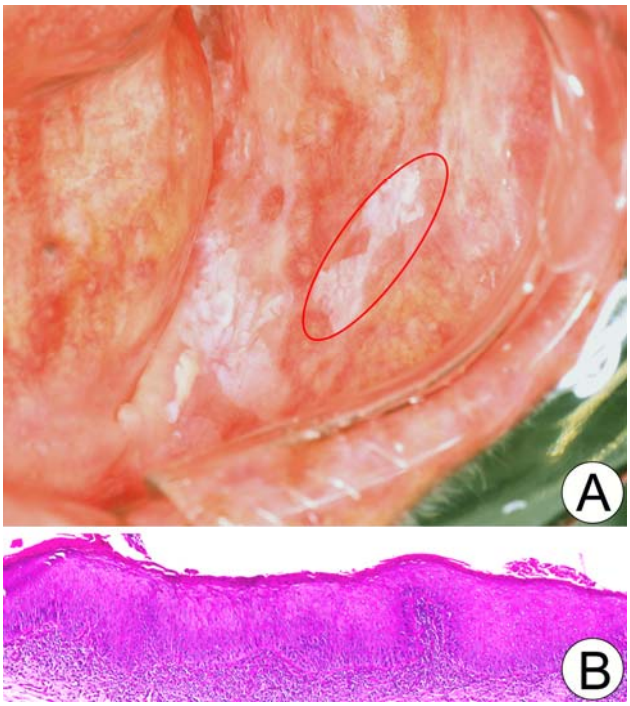

Figure 2: Leukoplakia on the lower lip mucosa **(A)**, depicting severe epithelial dysplasia (**B:** Hematoxylin and eosin stain x50).

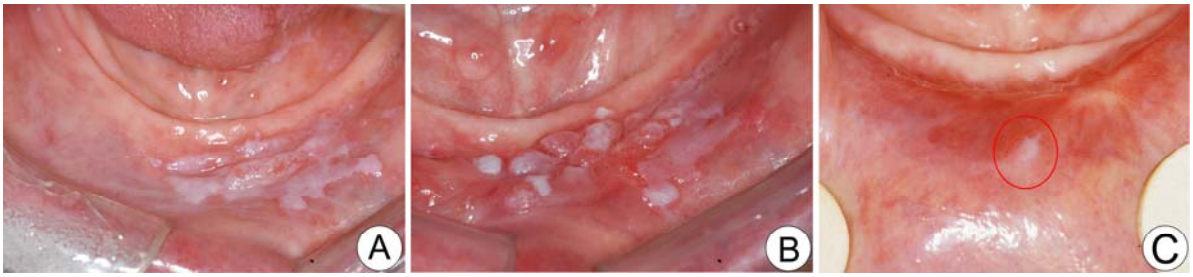

Figure 3: A PVL patient showing leukoplastic lesion on the left lower anterior fornix. **A –** This area showed superficial thickness and irregularity; **B –** The same site, one month later, presented a more prominent verrucous/multiple nodular surface. This area was excised; **C –** Ten months later: note the presence of a discrete leukoplakia near to the cicatricial area.

CAPÍTULO 4

PROLIFERATIVE VERRUCOUS LEUKOPLAKIA, SQUAMOUS CELL CARCINOMA AND AXILLARY METASTASIS

Running Title: Proliferative verrucous leukoplakia and axillary metastasis

Adriele Ferreira Gouvêa, DDS, MSc*
Adriana Elisa Brasil Moreira, MD**
Rodrigo Ribas Dias Reis, MD**
Oslei Paes de Almeida, DDS, PhD*
Márcio Ajudarte Lopes, DDS, PhD*

*Department of Oral Diagnosis – Oral Pathology Section, Piracicaba Dental School – University of Campinas, Piracicaba - São Paulo State, Brazil.
**Private office and Santa Casa Hospital, Piracicaba, São Paulo, Brazil

Corresponding author: Dr. Adriele Ferreira Gouvêa, DDS, MSc
Departamento de Diagnóstico Oral,
Faculdade de Odontologia de Piracicaba-UNICAMP
Av. Limeira, 901- Areião
Caixa Postal 52, CEP: 13414-903.
Piracicaba-SP, Brazil.
Phone: +55-19-21065320; FAX: +55-19-21065218.
E-mail: adrielefg@yahoo.com.br

ABSTRACT

Proliferative verrucous leukoplakia (PVL) is an aggressive form of oral leukoplakia with multifocal presentation, high rates of recurrence and malignant transformation. Although development of regional lymph node metastasis is relatively frequent in patients with oral squamous cell carcinoma, axillary metastasis is quite uncommon. This paper presents a case of a 64-year-old female patient who was diagnosed with PVL and developed five oral squamous cell carcinomas and later an axillary lymph node metastasis.

KEY WORDS: Proliferative verrucous leukoplakia, squamous cell carcinoma, axillary metastasis

INTRODUCTION

Proliferative verrucous leukoplakia (PVL) was first described by Hansen *et al.*, in 1985 (1) as a distinct and aggressive type of oral leukoplakia with unknown etiology affecting more commonly women in the sixth decade (female to male ratio = 4:1). PVL presents multifocal or diffuse extension, tendency to recur after treatment and high risk of malignant transformation (1-8).Several studies have reported high rates of malignant transformation which varies from 60% to 100% (2,3,9,10).

Metastasis from oral squamous cell carcinoma (SCC) follows a predictable way of lymphatic drainage that varies particularly according to the anatomic site, size and histopathological features (11). Eventually, metastasis can follow an unpredictable pattern and involve unexpected lymph nodes such as contralateral cervical lymph nodes, low jugular-carotid lymph nodal chain and even axillary lymph nodes (12,13).

There are few reports published in the English-language literature about axillary metastasis from head and neck squamous cell carcinoma (HNSCC) and fewer from oral SCC (14). This paper presents the case of a 64-year-old female patient who was diagnosed with PVL and developed five oral SCC and later an

axillary lymph node metastasis. To the best of our knowledge this is the first case report of axillary metastasis in a patient with oral SCC that developed from PVL.

CASE REPORT

A 64-year-old Caucasian woman, non-smoker, non-drinker, was referred to the Orocentro (Oral Diagnosis Clinic – Piracicaba Dental School - UNICAMP, Sao Paulo, Brazil) complaining of a tongue wound. She reported the onset of a white lesion on the tongue, four years before, which at that time was biopsied and showed extensive epithelial acanthosis and inflammatory infiltrate in the connective tissue. On intra-oral examination it was observed a very extensive leukoerythroplastic lesion with some verrucous and ulcerated areas on the dorsum and ventral surface of the tongue (Fig. 1 A). An incisional biopsy was performed and a well differentiated superficially invasive SCC was diagnosed (Table 1). A body screening was performed (nasofibrolaryngoscopy and chest X-ray) and no other neoplasia was detected. A left hemiglossectomy and left supraomohyoid neck dissection were performed, which showed no lymph node metastases. Because of the tumor size, the patient received post-operative radiotherapy to the left neck at a dose of 60Gy. Almost six months later, she presented leukoplastic lesions on the soft palate, tip and ventral surface of the tongue, right and left lateral border of the tongue, right buccal mucosa, right upper alveolar ridge and right inferior alveolar ridge and close periodical evaluation was decided. One month later all lesions remained stable but there was an erythroplastic area on the tip of the tongue and a biopsy was performed showing severe epithelial dysplasia (Table 1). All the clinically altered area was removed and the patient was closely followed. However, three months later a whitish lesion with granular surface on the tip and the right lateral border of the tongue developed. A biopsy involving this area was performed and a superficially invasive squamous cell carcinoma was diagnosed (Table 1, Fig. 1 B and Fig. 2 A). A body screening was performed (abdominal and mammary ultrasound, nasofibrolaryngoscopy and chest X-ray) and, again, no other neoplasia was detected. Thus, a right partial glossectomy was performed. One

year and five months after initial presentation, all lesions remained clinically stable with homogeneous surface, however another homogeneous leukoplastic area arose on the right retromolar area. After one year and seven months of follow-up, the right side of the floor of the mouth showed an ulcerated keratotic area. An extensive incisional biopsy was performed and the histopathological analysis detected mild dysplasia (Fig. 1 C) (Table 1). Almost eight months after this procedure the same area presented ulceration (Fig. 1 D). Another biopsy was performed and a well differentiated invasive SCC was diagnosed, being the patient submitted to another cancer surgery, after a complete body screening (Table 1). In the subsequent consultation, one month later, she presented new leukoplastic lesions on the buccal mucosa bilaterally and verrucous areas on the left inferior alveolar ridge and left posterior border of the tongue. The microscopical analysis of the latter showed severe dysplasia (Fig. 1 E). Therefore, she was referred to the surgical oncologist who removed the lesions. After three years of the first clinical evaluation, four months after the former management, the patient detected a 4 cm palpable lymph node on her right axilla, and then she was seen by her surgical oncologist. Biopsy of the lymph node was performed and the histopathological evaluation revealed a metastatic poorly differentiated SCC (Fig. 2 B). A primary breast carcinoma was suspected but there were negative findings on the mammogram. A body screening (abdominal and mammary ultrasound, nasofibrolaryngoscopy and chest X-ray) did not show any tumor. The patient was referred to the Orocentro and an aggressive lesion was identified on the right side involving lateral border of the tongue, floor of the mouth and alveolar ridge and another lesion on the left posterior lateral border of the tongue (Fig. 1 F). Biopsies of both areas were performed by her surgical oncologist and the microscopical analysis in both sites showed poorly differentiated invasive SCC (Table 1). Thus, one month later, the tumors were removed and a radical right axillary limphadenectomy was performed. Twenty-four lymph nodes were removed and just one level I lymph node presented metastatic SCC. Adjuvant chemotherapy was initiated (with cisplatin and fluoruracil), but because of the patient's systemic

condition just one cycle was performed. The patient developed pulmonary metastases two months after the chemotherapy cycle and died approximately six months after the removal of the tumors.

DISCUSSION

This very aggressive type of oral leukoplakia presents slow and persistent growth and high rates of malignant transformation (1-8). Batsakis *et al*. (1999) found SCC development occurrence in almost 100% of proliferative verrucous leukoplakia, an observation also corroborated by Silverman and Bagan, who found malignant transformation in 87% and 63,3% of PVL patients, respectively (3,4,9). Clearly, PVL is resistant to the available treatment modalities, such as cold knife surgery, CO_2.laser evaporation, laser surgery, chemotherapy, radiotherapy and presents frequent recurrences (8).

Distant metastases from head and neck SCC are relatively common, mainly after recurrence of local or regional disease, but metastases from the upper aerodigestive tract to the axillary lymph nodes is an unusual event (12,14). The metastases drainage way can be suggested mainly by location, size of the primary tumor and regional lymph nodal control (11). Under certain conditions, however, this drainage way may become unpredictable with some metastases bypassing the expected lymph node group (“skip metastases”), occurring at the contralateral side of the neck (cross-over of the lymphatic drainage) or even affecting axillary lymph nodes (12,13). Occurrence of axillary lymph nodes metastases in head and neck carcinoma is a very rare event, varying from 2-9%. Nevertheless, this incidence may be higher because impalpable lymph nodes sometimes are not detected (15,16). The axilla contains numerous lymph nodes, which follow along the axillary venous system and drain the anterolateral chest wall and the upper extremity. Complex and variable connections with lymphatic vessels of the chest and axilla do exist and, under certain conditions, the axillary lymph nodes can become the major lymphatic drainage site from the anterior and lateral neck (12). Changes in the lymphatic drainage can be promoted by a malignant tumor by metastatic blockage

of lymph nodes. Consequent fibrosis by surgical manipulation or radiotherapy is also another possible factor that leads to new lymphatic channels formation or aberrant lymphatic channels development (16). Also, there are some other explanations for axillary metastases from oral SCC: hematogenous dissemination; metastases from a second primary tumor along the aerodigestive tract; tumor dissemination after a paraestomal recurrence and retrograde dissemination due to blockage of the jugulo-subclavian junction (15). Our patient had a neck dissection, radiotherapy and recurrent disease, all possible factors involved in the alteration of the normal lymphatic net drainage that may lead to axillary metastasis.

The poor prognosis in cases with axillary metastasis may result from the high risk of simultaneous occurrence of other distant metastasis (16). Presentation of distant metastasis is usually in the late course of the disease and almost invariably means an unfavorable prognosis (11). Pulmonary metastases are the most frequent in HNSCC, accounting for 66% of distant metastases. Other sites include bone (hip, long bones or vertebral column), liver, skin, mediastinum and bone marrow (11). Patients with prior history of axillary metastases should have regular monitoring of their lymph nodes as part of their routine follow-up. Frequently palpation and, in suspicious cases, an ultrasound examination or CT should be done (16). Knowledge of this possibility is very important to reinforce the need of early recognition and timely surgically treat axillary metastasis before development of another distant metastasis, improving survival rates.

The present PVL case represented a management challenge, since the leukoplastic lesions presented rapid onset and progression, there was development of multiple SCC and latter on distant metastasis. Even with this impressive course, this case illustrates very well the PVL characteristics previously reported in the literature: resistance to all instituted treatments, frequent recurrences and high rates of malignant transformation. Although axillary metastasis from oral SCC is uncommon, this event was also observed in our patient, emphasizing the importance of close follow-up and careful examination.

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TABLES

Table 1: Summary of the lesions locations and histopathological findings.

Date	Biopsied sites	Histopathological findings
2002, November	Dorsum of the tongue and ventral of the tongue - left and right	SCC – poorly differentiated
2003, May	Tongue – tip	Severe epithelial dysplasia
2003, September	Anterior alveolar ridge and floor of the mouth – right	SCC
2004, June	Floor of the mouth – right	Acanthosis, hyperkeratosis, mild dysplasia
2005, March	Floor of the mouth – right	SCC
2005, August	Lateral posterior border of the tongue – left	Acanthosis, hyperkeratosis, severe dysplasia
2006, January	Floor of the mouth – right	Invasive poorly differentiated SCC
2006, January	Lateral border of the tongue – left	Invasive poorly differentiated SCC

Table 2: Summary of the chronological leukoplastic lesions development.

Date	Leukoplakic lesions locations										
	Alveolar ridge				Buccal mucosa		Tongue			Soft palate	Floor of the mouth
	Superior		Lower								
	Right	Left	Right	Left	Right	Left	Right	Left	Anterior		
2002 November							x	x	x		
2003 April	x		X		x		x	x	x	x	
2003 September							x		x		
2004 April			X								x
2005 April				x	x	x		x			x

FIGURES

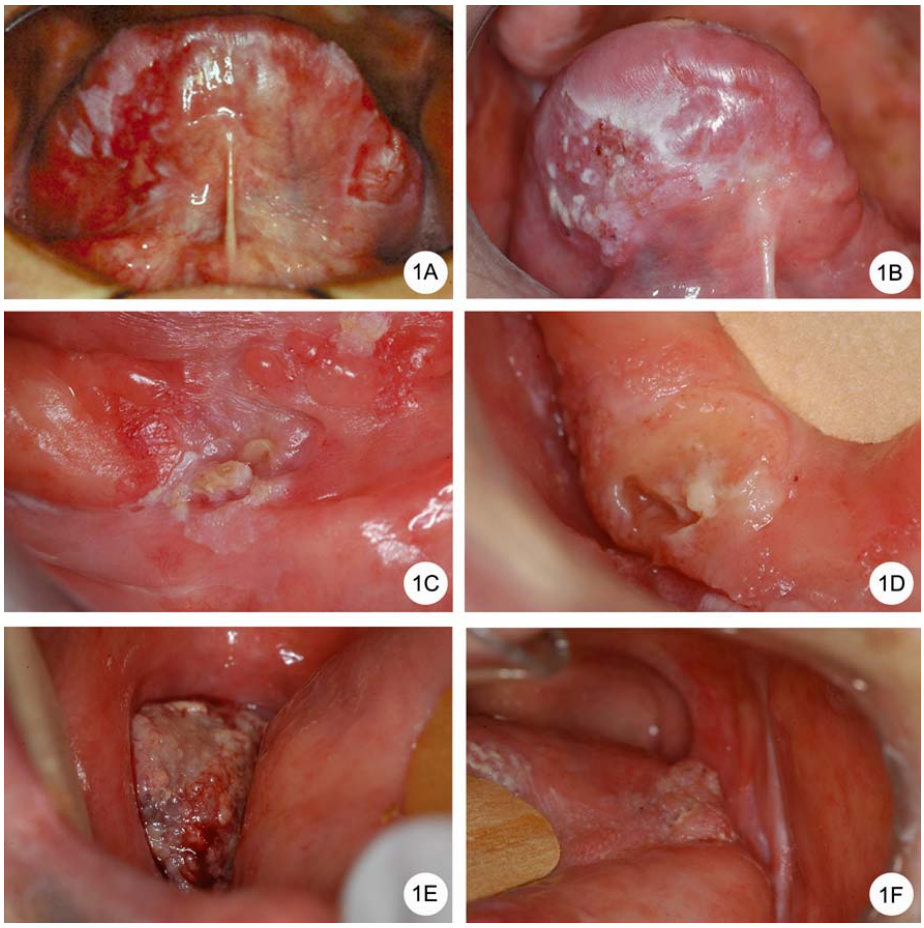

Figure 1

A. Patient's first clinical presentation: presence of an extensive leukoerythroplastic lesion with some irregular and verrucous areas involving the anterior dorsum and bilateral borders of the tongue.

B. A granular whitish area with ulcerated surface, on the right lateral border of the tongue involving the tip of the tongue and part of the floor of the mouth.

C. Lesion on the right anterior floor of the mouth presenting an irregular keratotic surface, which showed on the histopathological analysis mild epithelial dysplasia.

D. Ulcerated lesion on the right anterior floor of the mouth, diagnosed as squamous cell carcinoma.

E. Necrotic and invasive lesion on the right retromolar area, diagnosed as invasive poorly differentiated SCC.

F. Irregular and granular lesion on the left posterior border of the tongue, which on the histopathological analysis showed severe dysplasia. Later on, a SCC developed at this site.

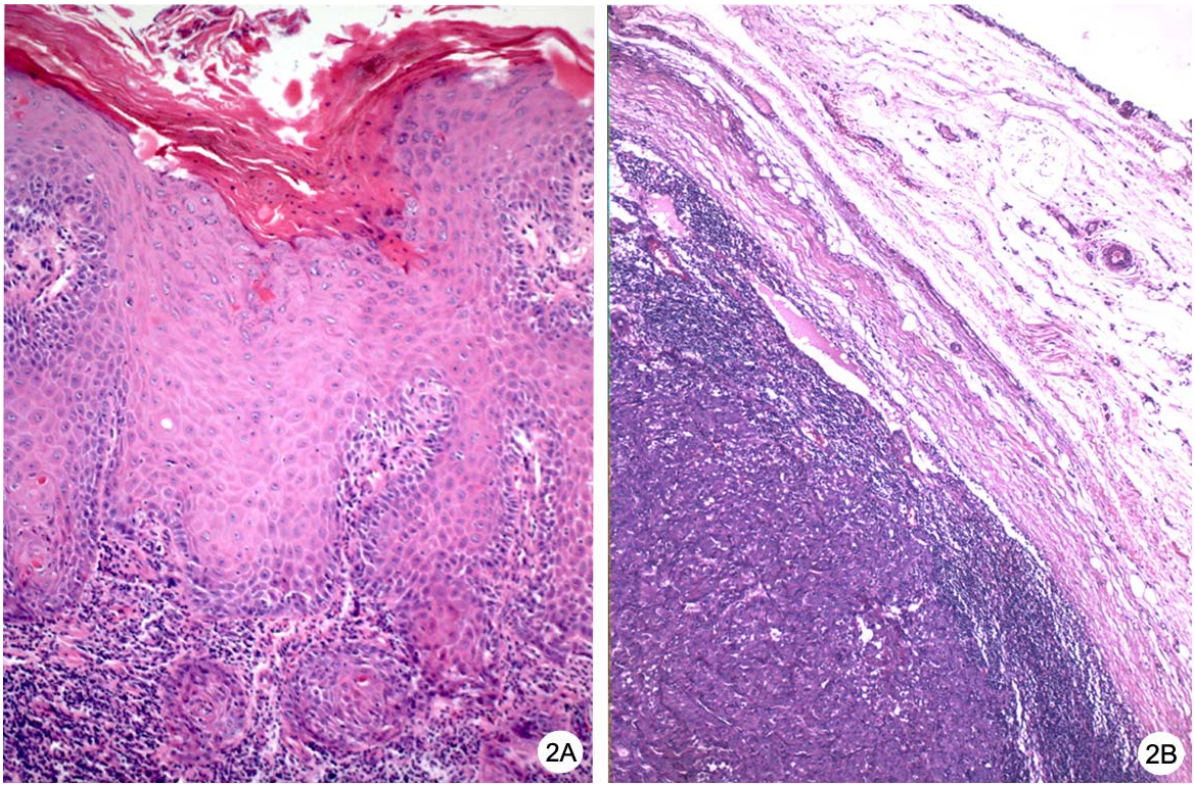

Figure 2

A. Microscopical aspect of the biopsy performed to the lesion shown on Fig 1 A, showing superficial invasion of SCC (Hematoxylin and eosin, original magnification ×200).

B. Microscopy of poorly differentiated metastatic SCC in the right axillary lymph node (Hematoxylin and eosin, original magnification ×50).

CONCLUSÕES

1. LVP é uma entidade distinta, com comportamento clinico singular e agressivo;

2. Alguns pacientes parecem apresentar lesões com comportamento mais indolente; com progressão clínica por anos a fio;

3. Análise imunoistoquímica demonstrou correlação entre expressão de Mcm2 e grau de displasia epitelial;

4. Análise da ploidia do DNA demonstrou que:

a) transformação maligna pode ser predita em grande número de casos (no presente estudo: quase 50% dos casos),

b) quase 90% dos tecidos biopsiados mostraram-se aneuplóides,

c) amostras apresentando leve alteração epitelial (hiperqueratose, acantose ou displasia epitelial leve) mostraram-se aneuplóides,

d) ocorre aumento da freqüência e do grau de aneuploidia conforme aumento do grau de alteração epitelial, assim como dos valores de DI, HI e frações excedentes de 5n.

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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Análise das características clínicas, histopatológicas, imunoistoquímicas e ploidia em leucoplasia verrucosa proliferativa**", protocolo nº **006/2009**, dos pesquisadores **ADRIELE FERREIRA GOUVÊA e MARCIO AJUDARTE LOPES**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 30/03/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Analysis of the clinical, histopathological and immunohistochemical characteristics and ploidy study of proliferative verrucous leukoplakia**", register number **006/2009**, of **ADRIELE FERREIRA GOUVÊA and MARCIO AJUDARTE LOPES**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 30/03/2009.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.