UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

FERNANDA VIVIANE MARIANO

ANÁLISE DAS CARACTERÍSTICAS CLINICOPATOLÓGICAS, PROLIFERAÇÃO CELULAR E ALTERAÇÕES DE NÚMERO DE CÓPIAS E METILAÇÃO DE GENES SUPRESSORES DE TUMOR EM CARCINOMA EX-ADENOMA PLEOMORFO

Tese de Doutorado apresentada a Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do Título de Doutor em Estomatopatologia, na Área de Patologia.

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(Isaac Newton)

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"Todos podem ser grandes
Porque qualquer pessoa pode servir
Não precisamos ter um diploma universitário para servir
Não temos de fazer concordância entre o sujeito e o verbo para servir
Não precisamos entender a teoria da relatividade de Einstein para servir
Precisamos apenas de um coração cheio de graça
Uma alma engendrada pelo amor”

Martin Luther King

RESUMO

O Carcinoma ex-adenoma pleomorfo (CXAP) é uma neoplasia indolente, cuja patogênese ainda não está esclarecida, embora se acredite que resulte do acúmulo de alterações genéticas em adenoma pleomorfo (AP) de longa permanência. No presente estudo, avaliamos os fatores clincopatológicos em uma série de 38 casos de CXAP provenientes de três instituições do estado de São Paulo, Brasil. Analisamos também, o índice de proliferação celular, através da imunomarcação de Ki-67 em 36 APs, 22 APs provenientes de CXAP e 36 CXAPs nas diferentes fases de progressão maligna (precoces e francamente invasivos), subdivididos quanto aos tipos histológicos, a fim de determinar uma possível ferramenta de auxílio diagnóstico. Com estes mesmos grupos de tumores, estudamos o perfil genético de ganho e perda de número de cópias e metilação de genes supressores de tumor, através da técnica de Multiplex Ligation Probe-Dependent Amplification (MLPA). Os resultados mostraram características clinicopatológicas semelhantes às descritas em grandes séries da literatura. Observamos que a marcação de Ki-67 pode ser uma útil ferramenta na distinção entre AP e CXAP, mesmo em fases precoces de transformação maligna. Este índice mostrou não ser importante para distinção dos subtipos histopatológicos. Além disso, encontramos várias alterações em genes supressores de tumor presentes durante a tumorigênese do AP e carcinogênese do CXAP, e observamos um aumento cumulativo de alterações genômicas, sendo algumas delas, específicas para cada fase.

Palavras chave: adenoma pleomorfo, carcinoma ex-adenoma pleomorfo, carcinogênese, características clinicopatológicas, genes supressores de tumor, Ki-67.

ABSTRACT

Carcinoma ex pleomorphic adenoma (CXPA) is an aggressive neoplasm, and its pathogenesis is still unclear, although it is believed to result from the accumulation of genetic alterations in pleomorphic adenomas (PAs) with long duration. In the present study, we evaluated the clinicopathological features in a series of 38 cases of CXPA from three institutions of the state of Sao Paulo, Brazil. We also analyzed the index of cell proliferation by labeling of Ki-67 in 36 PA, 22 PA from CXPA, and 36 CXPA in different stages of malignant progression (early and frankly invasive) subdivided in histolopathological types, to determine a possible tool to aid the diagnosis. With the same groups of tumors, it was studied also the genomic profile of gain and loss of copy number and methylation of tumor suppressor genes across Multiplex Ligation-Dependent Probe Amplification (MLPA). The results showed similar clinicopathological features to those described in large published series. We observed that Ki-67 is a useful tool in distinguishing between PA and CXPA, even in the early stages of malignant transformation. This index showed no importance for distinction among the several histological subtypes of CXPA. Furthermore, we find that various tumor suppressor genes are altered during PA tumorigenesis and CXPA carcinogenesis, and there is an accumulative increase of genomic alterations that seems to be specific for each phase.

Key words: pleomorphic adenoma, carcinoma ex-pleomorphic adenoma, carcinogenesis, clinicopathological features, tumor suppressor genes, Ki-67.

SUMÁRIO

INTRODUÇÃO

Carcinoma ex-adenoma pleomorfo (CXAP) é um tumor maligno raro, agressivo e com patogênese pouco entendida. Sua prevalência é de 5,6 casos por 100.000 neoplasias malignas e sua incidência é de 0,17 tumores por um milhão de pessoas Constitui aproximadamente 3,6% (0,9-14) das neoplasias de glândulas salivares, 11,6% (2,8 - 42,4) dos tumores malignos salivares e ocorre em 6,2% dos casos de pacientes portadores de adenoma pleomorfo (Gnepp & Wenig, 1991).

Manifesta-se geralmente na 6ª e 7ª décadas de vida e freqüentemente afeta a glândula parótida, mas pode desenvolver-se também na glândula submandibular e nas glândulas salivares menores, particularmente naquelas localizadas no palato mole e duro (Gnepp e*t al.,* 2005). Embora raros, há casos de CXAPs descritos em glândulas sublingual, lacrimal, mama, traquéia e cavidade nasal (Cho *et al*., 1995; Baredes *et al*., 2003; Hayes *et al.,* 2005; Ding *et al.,* 2007).

O tempo de evolução da doença é geralmente longo, com média de nove anos, variando de um mês a 52 anos e a apresentação clínica mais comum é aumento de volume envolvendo a área da glândula salivar acometida (Olsen & Lewis, 2001; Luers *et al.,* 2009). Na maioria dos pacientes afetados por CXAP, os indivíduos apresentam-se assintomáticos, podendo ocorrer dor quando há extensão do tumor para tecidos adjacentes (Zbaren *et al.,* 2008) e paralisia facial ou parestesia pelo envolvimento do nervo facial.

Macroscopicamente, nos casos descritos na literatura o tumor variava de 1 a 25 cm de diâmetro (Gnepp & Wenig, 1991; Lewis *et al.*, 2001). Histologicamente, para o diagnóstico de CXAP, é necessário encontrar áreas de adenoma pleomorfo (AP) em associação com um carcinoma, ou, excepcionalmente, somente o carcinoma, mas com histórico de que se desenvolveu como tumor recorrente no local prévio de um AP (Olsen & Lewis, 2001; Zbären *et al.*, 2008). O componente carcinomatoso pode representar 33% a 66% da massa tumoral (Lewis *et al.*, 2001; Zbaren *et al*., 2008).

Tanto as células epiteliais (luminais) quanto às células mioepiteliais (não-luminais) podem se malignizar no AP, porém, na maioria dos casos, o carcinoma origina-se das primeiras (Altemani *et al.,* 2005). Após a transformação maligna, diversos fenótipos carcinomatosos podem se desenvolver, sendo que o adenocarcinoma NOS (not otherwise specified) e carcinoma de ducto salivar são os mais freqüentes. Os outros tipos histológicos de carcinomas originados em APs são: epidermóide, indiferenciado, adenóide cístico,

células claras, mucoepidermóide, mioepitelial, epitelial-mioepitelial, células acinares, oncocítico, células basais e sarcomatóide (LiVolsi & Perzin, 1977; Tortoledo *et al.,* 1984; Olsen & Lewis, 2001). Acredita-se que o tipo histológico do carcinoma tenha influência no comportamento biológico do CXAP (Seethala*,* 2011). Alguns de nossos subtipos histopatológicos em nossa casuística estão ilustrados em anexo 4.

Os CXAPs são subclassificados quanto à invasão, considerando como referência a cápsula do adenoma, em: carcinoma intracapsular (contido pela cápsula e, portanto, não invasivo), minimamente invasivo (infiltração do tecido extracapsular a uma distância menor que 1,5 mm) e francamente invasivo (infiltração maior que 1,5 mm) (Brandewein *et al.,* 1996; Di Palma *et al.,* 2005; Gnepp *et al.,* 2005). No anexo 4, podem ser observados alguns exemplos de invasão. Os CXAPs intracapsular e minimamente invasivo tendem a exibir comportamento biológico de baixo grau, semelhante ao do AP com margens livres (LiVolsi & Perzin em 1977). Em contraste, o CXAP francamente invasivo apresenta comportamento mais agressivo, podendo dar origem a metástases, recidivas e levar o paciente à morte (Zbaren *et al*., 2008).

Diversos estudos têm buscado imunomarcadores que possam auxiliar na distinção das diferentes fases de invasividade do CXAP (Freitas *et al.,* 2005; Katori *et al.,* 2007; Katori *et al.,* 2007). Marcadores de proliferação celular são alvos constantes de investigação, visto que o índice de divisão celular aumenta com a progressão da invasividade. O antígeno Ki-67, também conhecido como MKI67, é uma proteína não histônica nuclear codificada pelo gene *MKI67,* localizado no braço longo do cromossomo 17 (Schonk *et al*., 1989; Bullwinkel *et al.,* 2006), sendo um dos mais importantes e estudados marcadores de proliferação celular.

A função da proteína Ki-67 está associada com proliferação celular e transcrição de RNA ribossomal (Rahmanzadeh *et al.,* 2007), e portanto, quando inativa, leva a inibição da síntese de RNA ribossomal (Scholzen *et al.,* 2000). Durante a intérfase, o antígeno Ki-67 pode ser exclusivamente detectada dentro do núcleo celular, onde em mitose, a maioria das proteínas são deslocadas para a superfície dos cromossomos. A proteína Ki-67 está presente durante todas as fases ativas do ciclo celular (G1, S, G2, M), mas ausente em G0 (Gerdes *et al.,* 1983).

Devido à sua função, é bem conhecido o papel do Ki-67 como valor prognóstico em diversos tipos de câncer, incluindo tumores malignos de glândulas salivares (Laitinen *et*

al., 2008; Vacchi-Suzzi *et al.,* 2010; Aune *et al.,* 2011; Habberstad *et al.,* 2011; Kapur *et al*., 2011; Luporsi *et al.,* 2011; Seethala *et al.,* 2011; Tang *et al.,* 2011; Yang *et al.,* 2011). Em CXAP, o índice médio de Ki-67 é usado para progressão e valor prognóstico (Di Palma *et al.,* 2005; Katori *et al.,* 2007; Katori *et al*., 2007; Seethala *et al.,* 2011). Além disso, o índice de Ki-67 pode ser uma ferramenta útil na distinção entre AP e CXAP, por causa do significante aumento de marcação na área carcinomatosa comparado à área de adenoma (Freitas *et al.,* 2005).

Com respeito ao tratamento do CXAP, a principal modalidade é a remoção cirúrgica. Ela é mais radical para casos francamente invasivos ou com acometimento do nervo facial (quando envolve a parótida) que para os casos intra-capsulares ou minimamente invasivos (Olsen & Lewis, 2001; Nouraei *et al.,* 2005). A necessidade de esvaziamento cervical ocorrerá nos casos com metástase regional. Terapia adjuvante como radioterapia pós-operatória é usada para doenças com alto grau de malignidade, em casos de margem comprometida, ou quando há metástase linfonodal, embolização vascular ou invasão perineural (Luers *et al.,* 2009). A combinação de radioterapia e quimioterapia é indicada para pacientes com doença disseminada (Olsen & Lewis, 2001; Luers *et al.,* 2009).

Recorrências locais e regionais ocorrem em 23% e 18% respectivamente e mudam drasticamente o prognóstico dos portadores de CXAP. Quando ocorrem, resultam em uma média de sobrevida de menos de um ano após descoberta (Olsen & Lewis, 2001). A doença metastática consiste somente de elementos carcinomatosos e quando ocorre à distância envolve principalmente pulmões e ossos.

As taxas de sobrevida dos portadores de CXAP variam entre os diferentes estudos. Por exemplo, Olsen & Lewis (2001) encontraram uma taxa de sobrevida livre de doença em 5 anos de 37% equivalente a 73 casos estudados, enquanto Zbaren *et al*. (2008) relataram sobrevida em 5 anos de 76% equivalente a 24 casos analisados. As diferenças podem ser explicadas pelas diferentes prevalências de tipos e graus histológicos dos carcinomas e níveis de infiltração.

Patogênese da transformação maligna do AP

Eneroth *et al.* (1968) estimaram que o carcinoma pode se desenvolver em 1,6-7,5% dos tumores mistos benignos não tratados. Análises de APs recorrentes indicam que cerca de 7,1% deles sofrem alterações malignas e o risco de malignidade provavelmente aumenta

com o tempo de evolução da doença (Phillips & Olsen, 1995). A patogênese da transformação do AP para CXAP ainda é pouco entendida, embora várias alterações genéticas e moleculares já tenham sido associadas com o processo de malignização do adenoma pleomorfo.

Analisando a carcinogênese em seu ponto inicial, sabe-se que o desenvolvimento e manutenção tumoral resultam do acúmulo de várias mutações cromossômicas e gênicas ocorridas em diversas vias e etapas de processos biológicos. Seguindo este raciocínio, pesquisadores têm focalizado seus estudos no descobrimento e mapeamento das alterações gênicas presentes em neoplasias malignas.

O primeiro estudo para o conhecimento e compreensão da progressão maligna de PAs foi publicado pela Eneroth & Zetterberg (1974). Eles fizeram a análise de ploidia em PAs com duração de evolução clínica menor que um ano e encontraram uma população diplóide. Em contrapartida, nos PAs com mais de cinco anos de evolução, observou-se uma população tetraplóide, similar aos casos de CXPAs, sugerindo que o crescimento tumoral e modificações genéticas ocorrem simultaneamente com a transformação maligna. Outros estudos posteriormente confirmam esses achados (Jin *et al*, 1994;. Brandewin *et al*, 1996;. Martins *et al*, 1996;. El-Naggar *et al,* 1998;. El-Naggar *et al*, 2000;. Lewis *et al*., 2001; Vargas *et al.,* 2007).

O estudo da carcinogênese ao longo do tempo tem mostrado que o desenvolvimento e manutenção do tumor, resulta não só do crescimento tumoral e alterações genéticas, mas sobretudo da acumulação de várias mutações genéticas e cromossômicas ocorrendo em várias vias da biologia do câncer. Seguindo este raciocínio, as pesquisas têm focado suas investigações em alguns delas, que incluem (1) auto-suficiência em sinais de crescimento, (2) insensibilidade aos sinais inibitórios de crescimento (anticrescimento), (3) a incapacidade de morte celular programada (apoptose), (4) potencial replicativo ilimitado, (5) angiogênese sustentada, e (6) a invasão de tecidos e metástase (Hanahan & Weinberg, 2000).

A capacidade proliferativa celular autonoma é uma das mudanças adquiridas pelas células neoplásicas que ocorre por meio da ativação genética de oncogenes e inativação de genes supressores de tumor (Vogelstein *et al*., 2004). Os genes supressores de tumor têm papel importante no desenvolvimento e progressão da CXPA, decorrente de sua inativação, seja por mutação, deleção ou metilação.

Fowler *et al*. (2006) identificaram uma significante taxa de deleção por perda de heterozigosidade em CXAP associada à região dos genes supressores de tumor: *P53*, *NM23-H1*, *DCC*. Perda de heterozigosidade do gene *P53* também foi demonstrada por Yamamoto *et al.* (1996). Os autores ainda citam este mesmo tipo de mutação nos genes *K-ras* e *RB*. Gedlicka *et al*. (2010) em contrapartida não encontraram mutação do gene *TP53* através de análise feita com seqüenciamento. Outros genes investigados na carcinogênese do CXAP, porém sob o ponto de vista de metilação foram: *p16, CYGB, RASSF1, RARb, hTERT, WT1, TMEFF2*. De todos, Schache *et al*. (2010) encontraram uma relação estatisticamente significativa entre metilação de *RASSF1* e progressão para CXAP. Com uma significância menor, porém também metilados, foram identificados os genes: p16, *WT1, hTERT,* implicando também função na carcinogênese do CXAP. Hu *et al.* (2011) também observaram que a metilação do gene *P16* pode estar correlacionada à transformação maligna do AP.

A metilação tem sido detectada em uma alta porcentagem de tumores diferentes e é um alvo interessante para diversos estudos, porque o silenciamento de genes por mudanças epigenéticas são potencialmente reversíveis e, portanto, o fenômeno é atraente para o desenvolvimento de novas abordagens terapêuticas (Miracca *et al.,* 1999). No entanto, há poucos estudos associados a metilação em CXAP e muitos genes supressores tumorais envolvidos no processo de transformação carcinomatosa precisam ser melhor investigados.

Tendo por base os dados revistos na literatura, permanecem muitas questões sobre a carcinogênese do CXAP. Deste modo, o primeiro objetivo do presente trabalho é apresentar apresentar dados descritivos da primeira significativa série Brasileira composta por 38 casos de CXAP. O segundo objetivo é analisar o grau de proliferação celular durante a transformação maligna do AP, progressão do CXPA e comparar os diferentes subtipos histopatológicos, através do índice proliferativo por Ki-67, podendo-se obter uma ferramenta diagnóstica. O terceiro objetivo é apresentar um perfil genômico de perda, ganho e metilação de um painel de genes supressores de tumor Multiplex Ligation Probe-Dependent Amplification (MLPA) em um grupo de AP e CXAP.

CAPÍTULO 1

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CARCINOMA EX-PLEOMORPHIC ADENOMA IN A BRAZILIAN POPULATION: CLINICOPATHOLOGIC ANALYSIS OF 38 CASES

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ABSTRACT

INTRODUCTION: Carcinoma ex-pleomorphic adenoma (CXPA) is a rare tumor with different prevalence among studies. There are few epidemiological studies of large series of CXPAs in developing countries. **OBJECTIVE:** The aim of the present study is to present a

Brazilian retrospective study of 38 patients with CXPA. **MATERIAL AND METHODS:** Thirty and eight cases of CXPA from three Brazilian institutions, São Paulo State were evaluated. The patients were analyzed according to gender, age, location, clinical stage, histopathological subtype, invasiveness of the tumor, and follow up information. **RESULTS:** No predilection gender was found, with mean age of 57.6 years. The most involved site was parotid (73.7%), followed by the submandibular (15.8%), and minor salivary gland (10.4%). At diagnosis 9 (23.6%) were classified as clinical stage I and II, 19 (50.1%) stage III and IV, and 10 (26.3%) without information. The histological types were salivary duct carcinoma (42.2%), followed by adenocarcinoma not otherwise specified (21.2%), myoepithelial carcinoma (18.4%), epithelial-myoepithelial carcinoma (13.2%), epidermoid carcinoma (2.6%), and sarcomatoid carcinoma (2.6%). Moreover, according to invasive phase, 15.8% were classified as intracapsular, 23.7% minimally invasive and 60.5% frankly invasive. Recurrence was observed in 18.4% frankly invasive cases during the follow-up. During follow-up 13.1% of the patients died of causes related to the disease. **CONCLUSION:** Distribution of cases according to age, gender, tumor location and clinical stage were similar to the reposted literature. Frankly invasive cases presented worse prognosis. Besides current study, more information are need to understand the clinicopathologic aspects of CXPA.

Key words: Carcinoma ex-pleomorphic adenoma, series, histopathological subtypes, invasiveness.

INTRODUCTION

Carcinoma ex Pleomorphic Adenoma (CXPA) is malignant tumor of salivary gland that comprises 3% of all salivary tumors, 6.2% of all pleomorphic adenomas (PA), and 11.7% of all salivary malignancies. The CXPA is uncommon, with a prevalence rate of 5.6 cases per 100,000 malignant neoplasms and an incidence rate of 0.17 tumors per 1 million persons[1,2]. It is considered to be a malignant transformation of a pre-existing pleomorphic adenoma, resultant of the accumulation of tumor-associated genetic alteration of normal cells[3]. About 1.6–7.5% of pleomorphic adenoma have malignant changes in its natural course[4,5].

The most common site of origin is the parotid gland; but it may originate from submandibular gland and minor salivary gland sites, mainly in soft and hard palate[2]. Although rare, there are CXPAs cases described in sublingual gland, lacrimal gland, breast, trachea and nasal cavity[6-9].CXPA usually presents in the 6th or 7th decades of life with female predilection[10].

The CXPA may be classified as intracapsular, minimally invasive and frankly invasive. The main recommended treatment for CXPA is wide local excision. Adjuvant radiation therapy can be used for cases of invasive tumors, positive margins, vascular or neural invasion[2].

The reported incidence of CXPA tumors is generally low and varies considerably from series to series. The largest series previously reported have included 57 cases described by Foote & Frazell (1953)[11], 29 by Beahrs *et al*. (1957)[12], 32 by Moberger & Eneroth (1968)[13], 25 by Gerughty *et al.* (1969)[14], 47 by LiVolsi & Perzin (1977)[15], 146 by Spiro *et al*. (1977)[16], 40 by Tortoledo *et al.* (1984)[17], 102 by Eveson & Crawson (1985)[18], 326 by Gnepp & Wenig (1991)[1], 73 by Lewis *et al*. (2001)[19], 24 by Zbaren *et al*. (2008)[20], 43 by Katabi (2010)[21], all of which are North American and Europe populations. Hence, the aim of this paper is to present a developing country retrospective clinicopathologic analysis involving 38 patients diagnosed with CXPA.

MATERIAL AND METHODS

A retrospective review of medical records was performed and included 38 eligible patients with salivary gland carcinomas diagnosed as CXPA from at Hospital of the University of Campinas (UNICAMP), AC Camargo Hospital and Piracicaba Dental School (UNICAMP), São Paulo State, Brazil. This study was approved by the Ethics Committee of each institution. The medical records of patients were examined for demographic data (age, gender) and clinical information (duration of the symptoms, medical history, clinical stage). TNM restaging was reviewed according to the International Union Against Cancer criteria (UICC, 2002)[22]. Treatment, recurrence and follow-up data were also recovered.

The histology of the 38 cases of CXPA was reviewed using 5 µm hematoxylin-eosin-stained sections of formalin-fixed, paraffin-embedded samples. In the cases of CXPA, the malignant area was distinguished from the PA based on morphology and architectural atypia. The histopathological review was done in according to World Health

Organization (WHO, 2005)[23]. Microscopical examination of PA showed a well-circumscribed lesion, with intense hyalinization or myxoid areas, clusters and nests of epithelial cells, ductal structures composed of epithelial and myoepithelial cells. There was no pleomorphism, cellular atypia or mitosis. Nevertheless, the microscopical examination of CXPA showed pleomorphic cells with increased nucleus/cytoplasm ratio, hyperchromatic nuclei and prominent nucleoli. All the cases of CXPA were classified according to invasiveness (intracapsular, minimmally invasive, frankly invasive phase) and histopathological subtypes.

RESULTS

The studied population consisted of 38 patients with CXPA, of which 19 were male and 19 female with a mean age of 57.6 years (ranging from 27 to 88). The mean time of complaints was 115 months (ranging from 2 to 360), and three (7.9%) had the malignant tumor as recurrence of PA, after 5, 11 and 16 years. The most common symptom was tumor growth (35 cases, 92.1%). Three patients complaint of pain, one reported facial paralysis, one headache, one otalgia and one had salivary flow alteration. Most tumors involved the parotid (28 cases, 73.7%), followed by the submandibular (6 cases, 15.8%), and minor salivary glands of the oral cavity (2 palate, 1 upper lip – 7.8%) and one in minor mucous gland from nasal cavity (2.6%).

The mean tumor size was 5 cm (ranging from 1.5 to 9.5), with 6 patients (15.7%) showing invasion of adjacent structures such as skin, bone, and muscle. At diagnosis, 17 patients (44.7%) presented tumors in an advanced clinical stage (T3 or T4 tumors), 11 cases (29%) were classified as T1 or T2, and 10 (26.3%) had not information. In 10 (26.3%) it was not possible to get this information. Only 6 cases (15.8%) had lymph node metastasis at presentation, and 1 patient (2.6%) had distant metastasis involving the lungs. The final clinical stage of the cases that could be classified showed that 9 cases (23.6%) were classified as clinical stage I and II, 19 (50.1%) stage III and IV, and 10 (26.3%) had not information. The clinical parameters are described in the **Table 1**.

The most common histological type was salivary duct carcinoma (SDC) with 16 cases (42.2%), followed by adenocarcinoma not otherwise specified (AdNOS) 8 cases (21.2%), myoepithelial carcinoma (MC) 7 cases (18.4%), epithelial-myoepithelial carcinoma (EMC) 5 cases (13.2%), epidermoid carcinoma (EC) 1 case (2.6%), and

sarcomatoid carcinoma (SC) 1 case (2.6%). The invasiveness of the CXPA was subdivided into intracapsular (without invasion), minimally invasive (<1.5 mm) and frankly invasive phase (>1.5 mm). Six cases (15.8%) were classified as intracapsular, 9 (23.7%) minimally invasive and 23 (60.5%) frankly invasive phase.

Patients were treated mainly by surgery (30 cases - 79%) and 8 (21%) had not information. The adjuvant radiotherapy was realized in 7 cases (18.4%), due positive surgical margins. No patient was submitted to chemotherapy. Eight patients (21.1%) underwent neck dissection due positive clinically palpation and 5 of these patients (62.5%) had pathologically positive lymph nodes (pN+).

Seven patients (18.4%) experienced tumor recurrences during the follow-up (from 1 to 80 months) and all were frankly invasive CXPA. Of these 7 patients, 1 (2.6%) had local recurrence, 1 (2.6%) regional recurrence, 2 (5.2%) distant metastasis (lung and another site without specification), 1 (2.6%) local and regional recurrences, and 1 (2.6%) local and distant (orbital site) recurrences. A total of 14 patients (36.9%) were lost to follow up and there was no documentation of recurrences or current medical status, because they were referred for the pathology department only for diagnosis and were not treated at one of the participating institutions. The mean follow-up time was 29 months, ranging from 1 to 115 months. During follow-up period, 11 patients (29%) were alive without evidence of disease, 7 patients (18.4%) died of causes related to the disease and 6 patients (15.8%) died of other causes not related to the disease. The cases are summarized in the **Table 3.**

DISCUSSION

The CXPA is an infrequent tumor that has been studied over time and named as malignant mixed tumor, carcinoma ex mixed tumor, carcinoma ex adenoma, carcinoma ex benign pleomorphic adenoma, and CXPA is widely since half of the twentieth century[2].

Geographical differences in the prevalence of CXPA have been reported in the literature. Eveson & Crawson (1985)[18] noted that CXPA forms about 11.7% of British Salivary Gland Tumor Panel in 1975 (2,410 cases). Zbären *et al.* (2008)[20] suggested that CXPA comprises 14% of all primary parotid malignant neoplasms over a 20 year period in Switzerland. A 30-year Memorial Hospital (USA) experience, from 1939 to 1968, there was 146 patients with malignant mixed tumor that comprised 5% of 2,743 patients treated for salivary neoplasms (Spiro *et al.,* 1977)[16]. Lewis *et al.* (2001)[19] presented 73 cases of

CXPA of major salivary glands from Mayo Clinic files in a period of 34 years (ranging 1960 to 1994). Vargas *et al.* (2002)[24] found 3 cases of CXPA out 124 salivary gland tumors in a period of six years in Brazil, representing 2.4%. Altemani *et al*. (2005)[25] also in Brazil, studied 16 cases of CXPA, but without clinicopathological features. Hence, to the best of the authors' knowledge, this is the largest series of CXPA in a Brazilian population.

Gerughty *et al.* (1969)[14] believed these tumors were malignant from the onset, because 60% of the patients in their series were initially seen without a history of a preexisting tumor. Beside that the tumors occurred in young patients, and there was no appreciable difference between the age at onset in their series and series with benign mixed tumors. In contrast, Beahrs *et al.* (1957)[12] suggested that a carcinomatous transformation of a benign mixed tumor occurred, because the median age at onset for benign mixed tumors was 10 years younger than that for CXPA, and most patients were initially seen with a history of a mass present for many years. These features are the most often observed in subsequent series.

The risk of malignancy of PA varies from 1.6% to 7.5% in according to Eneroth (1964)[4], Eneroth *et al*. (1968)[5]. Analysis of recurrent PAs indicate that 7.1% of them undergo malignant changes and the risk of malignancy increases with time of disease progression (Phillips & Olsen, 1995)[26]. We observed that three cases of our series arose as recurrent PA. Katabi *et al.* (2010)[21] also found three cases of CXPA arose as recurrent PA.

Spiro *et al*. (1977)[16] found 78 women (54%) and 68 men (46%), with mean age of 54 years. The mean age of the patients was 60.8 years with slight female predilection in study of Eveson & Cawson (1985)[18]. Katabi *et al*. (2010)[21] found a median age of 59 years, with 63% in female gender and 37% in male gender. Our 38 cases of CXPA showed no predilection gender, with a mean age of 57.6 years. Differently, Lewis *et al.* (2001)[19] analyzed 73 cases of CXPA, of which 64% occurred in males and 36% in females, with mean age of 61 years.

The parotid gland is the main site affected by CXPA and submandibular or minor salivary gland involvement is rare. The majority of patients present with a rapidly growing painless mass in the last months before the diagnosis[1,2], but pain, facial nerve palsy and skin fixation may also occur[1,15]. Our study showed that the most tumors were painless involving the parotid (28/38), followed by the submandibular (6/38), and minor salivary glands (4/38). Only three patients complaint pain and one with facial paralysis.

Lewis & Olsen (2001)[19] observed a mean of tumor size of 3.9 cm, ranged from 1 to 17 cm. An important invasion into adjacent structures was found (64%). Metastases regional and distant were reported in 49% and 4% at the time of diagnosis. Most patients (64.4%) presented advanced stage of disease, III or IV. The current study showed a mean size of 5 cm, ranged from 1.5 to 9.5 cm, and only 15.8% presented invasion to adjacent tissues. Six cases (15.8%) had lymph node metastasis and one (2.6%) had distant metastasis. Nevertheless most patients had tumors that were classified as clinical stages III and IV.

According Gerughty *et al*. (1969)[14] CXPA is a difficult to diagnose, because the mixed tumor component is often small and overlooked, and the malignant component may be difficult to classify. The carcinoma is most frequently a poorly differentiated adenocarcinoma, SDC, or AdNOS, but different findings are found[2]. Lewis *et al*. (2001)[19] found in their series, 31 AdNOS, 24 SDC, 5 adenosquamous, 3 UC, 3 adenoid cystic carcinoma (ACC), 3 MC, 1 EMC, 1 SC. Tortoledo *et al*. (1984)[17] found SDC as the most frequent malignant subtype, and none of the malignancies were classified as AdNOS. Zbaren *et al*. (2008)[20] found AdNOS and ACC as the subtype histopathological most common, followed by mucoepidermoid carcinoma and SDC. Our samples presented high and medium grade of malignancy. The most commonly found histological subtype in descending order were: SDC (16 cases), AdNOS (8 cases), MC (7 cases), EMC (5 cases), SC (1 case), and EC (1 case).

It was observed that most cases of SDC and MC were clinically classified as stages III and IV. The AdNOS had the highest prevalence in I and II stage. Six cases (15.8%) were classified as intracapsular, 9 (23.7%) minimally invasive and 23 (60.5%) frankly invasive phase. All the seven cases that development recurrences were frankly invasive CXPAs, and five out these died for reasons related to disease (2 SDC, 1 AdNOS, 1 MC, 1 EMC). Katabi *et al*. (2010)[21] found regional and distant metastasis arose early invasive CXPAs, showing that even having biological course less aggressive, they can recur and cause death.

Surgery was the main modality of treatment of our cases and series described. Some cases underwent to neck dissection, due palpable lymph node or advanced stage disease. Few cases underwent adjuvant radiotherapy, due positive margin. Despite the aggressiveness of CXPA, it was observed a low rate of death (13.1%). But we do not have follow up information of about one third of the diagnosed cases.

In conclusion, our series revealed several malignant phenotypes with a similar rate of recurrence and death. The frankly invasive cases presented the worse prognosis. The age and gender predilection were similar to literature. Hence, this Brazilian series can contribute with better clinicopathologic knowledge about CXPAs.

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TABLES

Table 1- Clinical parameter of the 38 cases of CXPA.

VARIABLE	CATEGORY	N (%)
Gender	Male	19 (50)
	Female	19 (50)
Age	≤ 50	10 (26.3)
	≥ 51	24 (63.2)
	Information not available	4 (10.5)
	Mean	57.6
Disease history	Primary	35 (92.1)
	RecurrentPA	3 (7.8)
Site	Parotid	28 (73.7)
	Submandibular gland	6 (15.8)
	Minor salivary gland	4 (10.5)
Tumor size	T1	4 (10.5)
	T2	7 (18.4)
	T3	13 (34.3)
	T4	4 (10.5)
	Information not available	10 (26.3)
Clinical stage	I	4 (10.5)
	II	5 (13.2)
	III	14 (36.8)
	IV	5 (13.2)
	Information not available	10 (26.3)

Table 2- Histopathological subtypes and correlations of the 38 cases of CXPA.

		HISTOPATHOLOGICAL SUBTYPES						
Variable	**Category**	**SDC**	**AdNOS**	**MC**	**EMC**	**EC**	**SC**	**TOTAL**
Site	Parotid	12	6	4	4	1	1	28
	Submandibular gland	4	1	1	0	0	0	6
	Minor salivary gland	0	1	2	1	0	0	4
T stage	T1	1	2	0	1	0	0	4
	T2	1	2	3	1	0	0	7
	T3	7	2	2	2	0	0	13
	T4	1	1	1	0	0	1	4
	INA	6	1	1	1	1	0	10
Clinical stage	I	1	2	0	1	0	0	4
	II	0	2	2	1	0	0	5
	III	8	2	2	2	0	0	14
	IV	1	1	2	0	0	1	5
	INA	6	1	1	1	1	0	10
Type	Intracapsular	3	2	1	0	0	0	6
	Minimally invasive	6	1	1	1	0	0	9
	Frankly invasive	7	5	5	4	1	1	23
Recurrence	Yes	3	1	1	1	1	0	7
	No	6	5	3	2	0	1	17
	INA	7	2	3	2	0	0	14
Clinical status	Alive without disease	2	4	3	1	0	1	11
	Died of disease	3	1	1	1	1	0	7
	Died of other causes	4	1	0	1	0	0	6
	INA	7	2	3	2	0	0	14

SDC=Salivary duct carcinoma; AdNOS=Adenocarcinoma not otherwise specified; MC=Myoepithelial carcinoma; EMC=Epithelial-myoepithelial carcinoma; EC=Epidermoid carcinoma; SC=Sarcomatoid carcinoma; INA=Information not available.

Table 3- Summary of CXPAs cases.

Age	Gender	Site	Time duration	pT (cm)	T	N	M	Estadio	Histopathological subtype	Type	Treatment	Recurrence	Current time	Follow up
60	M	Parotid	25 Y	8	T3	N0	M0	III	SDC	IC	Surgery	No	DOC	115 M
61	F	Parotid	20 Y	5	T3	N0	M0	III	MC	IC	Surgery, RT	No	AWD	36 M
59	F	Parotid		5	T3	N0	M0	III	SDC	IC	Surgery			
37	F	SMG	1 Y	5,5	T3	N0	M0	III	SDC	IC	Surgery			
66	M	SMG	1 Y	2	T1	N0	M0	I	AdNOS	IC	Surgery	No	AWD	12 M
69	M	MSG	4 Y	2	T1	N0	M0	I	AdNOS	IC	Surgery	No	AWD	27 M
30	F	Parotid	2 Y	7,5	T3	N0	M0	III	EMC	MI	Surgery	No	AWD	1 M
73	M	Parotid	15 Y	4,5	T3	N0	M0	III	SDC	MI	Surgery	No	DOC	47 M
39	F	SMG		4	T2	N0	M0	II	MC	MI	Surgery			
57	F	Parotid							SDC	MI	Surgery			
57	F	Parotid	10 Y						SDC	MI	Surgery			
88	F	SMG	4 M	2	T1	N0	M0	I	SDC	MI	Surgery, RT	No	DOC	45 M
64	M	Parotid				N0	M0		SDC	MI	Surgery			
56	M	Parotid	6 Y	4	T2	N0	M0	II	AdNOS	MI	Surgery	No	AWD	3 M
54	M	Parotid	1 Y	4,5	T3	N0	M0	III	SDC	MI	Surgery	No	DOC	3 M
45	M	Parotid	4 Y	6	T3	N0	M0	III	SDC	FI	Surgery	Local, RM	DOD	85 M
50	F	Parotid	20 Y	8	T3	N1	M0	III	EMC	FI	Surgery,ND (pN-)			
74	F	MSG	30 Y	4	T2	N0	M0	II	MC	FI	Surgery	Local, DM (eyes)	DOD	24 M
	F	Parotid							MC	FI	Surgery			
65	M	Parotid	2 Y	8	T4a	N1	M0	IVa	SDC	FI	Surgery, ND (pN+)	No	AWD	6 M
82	F	Parotid	20 Y	6,2	T4a	N0	M0	IVa	SC	FI	Surgery, RT	No	AWD	24 M
27	F	Parotid		4	T2	N0	Lung	IVc	MC	FI	Surgery			
	F	Parotid	10 Y						EC	FI	Surgery	DM (lung)	DOD	
86	F	SMG							SDC	FI	Surgery			
62	F	SMG	18 Y			N1			SDC	FI	Surgery, ND (pN+), RT	DM	DOD	12 M
66	F	Parotid		1,5	T1	N0	M0	I	EMC	FI	Surgery			
64	M	Parotid	20 Y	4	T2	N0	M0	II	EMC	FI	Surgery	No	DOC	45 M
	M	Parotid							SDC	FI	Surgery			
56	F	MSG	5 Y						EMC	FI	Surgery	Yes	DOD	48 M
72	M	Parotid	2 Y	9,5	T4a	N1	M0	IVa	MC	FI	Surgery, ND (pN-), RT	No	AWD	58 M
66	M	Parotid	7 M	6	T3	N1	M0	III	SDC	FI	Surgery, ND (pN+), RT	RM	DOD	8 M
54	M	Parotid	4 Y	2,8	T3	N0	M0	III	AdNOS	FI	Surgery	No	AWD	12 M
48	M	MSG	10 Y	4,8	T3	N0	M0	III	MC	FI	Surgery	No	AWD	1 M
	M	Parotid							AdNOS	FI	Surgery			
41	M	Parotid	15 Y	6	T4a	N0	M0	IVa	AdNOS	FI	Surgery,ND (pN-)	No	DOC	9 M
51	M	Parotid	2 M	7	T3	N0	M0	III	AdNOS	FI	Surgery, ND (pN+), RT	Local	DOD	22 M
41	M	Parotid	16 Y	3,5	T2	N1	M0	III	SDC	FI	Surgery, ND (pN+)	No	AWD	22 M
39	F	Parotid		3,5	T2	N0	M0	II	AdNOS	FI	Surgery			

DM=Distant metastasi; RM=Regional metastasis; EMC=Epithelial-myoepithelial carcinoma; MC=Myoepithelial carcinoma; SDC=Salivary duct carcinoma; SC=Sarcomatoid carcionoma; EC=Epidermoid carcinoma; MSG=Minor salivary gland; SMG=Submandibular gland; IC=Intracapsular; MI=Minimally invasive; FI=Frankly invasive; ND=Neck dissection; Y=Years; M=Months; DOD=Died of disease; DOC=Died of other causes; AWD=Alive without disease.

CAPÍTULO 2

Artigo em preparação

ANALYSIS OF CELLULAR PROLIFERATION INDEX IN 36 CASES OF CARCINOMA EX-PLEOMORPHIC ADENOMA

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ABSTRACT

INTRODUCTION: Carcinoma ex-pleomorphic adenoma (CXPA) is a rare and aggressive malignancy of the salivary glands that arises from a previous PA. CXPA present different

histological subtypes and grade of invasiveness, leading to variable clinical behavior. **OBJECTIVE:** Determine the proliferative index of 36 cases of CXPA, considering the histological subtypes and invasiveness phase. **MATERIAL AND METHODS:** It was included on this study 36 each of CXPA and PA, and 22 areas of PA in CXPA. All cases of CXPA were classified according to invasiveness (early and frankly invasive phase) and histological subtype. Proliferative index was determined by Ki-67, and the data were statistically analyzed by Wilcoxon's, Mann-Whitney and Kruskal-Wallis' tests. **RESULTS:** CXPA included 14 cases classified as early invasive and 22 as frankly invasive phases. Fifteen cases corresponded to salivary duct carcinoma, 7 adenocarcinoma NOS, 7 myoepithelial carcinoma, 5 epithelial-myoepithelial carcinoma, one each squamous cell carcinoma and sarcomatoid carcinoma. The Ki-67 mean index of PA and residual PA were 6.7±2.5 and 6.9±8.2, respectively, significantly lower than CXPA (49.3±21.7). Early invasive carcinoma (44.3±23.0) showed smaller proliferative index than frankly invasive (52.5±20.8). Considering the subtypes of CXPA, there was not a statistic difference among them. **CONCLUSION:** Ki-67 is a useful marker in the differential diagnosis of PA and CXPA, even when in the early invasive phase. Ki67 index varies according to the histological subtype of CXPA, but are not statistically significant.

Key words: Pleomorphic adenoma, carcinoma ex-pleomorphic adenoma, Ki-67, invasiveness, histopathological subtypes, malignant progression.

INTRODUCTION

Carcinoma ex-pleomorphic adenoma (CXPA) is a rare malignant tumor that affects the salivary glands, corresponding to 3.6% of all salivary neoplasms and 11.7% of all salivary malignancies. It arises from a previous PA, usually in the 6th and 7th decades of life, as a slow painless growing mass (Gnepp *et al*., 2005). It is an interesting model of carcinogenesis, presenting various histological subtypes.

CXPA is classified according with invasiveness into non-invasive, minimally invasive and frankly invasive, with the latter showing a worse prognosis (Olsen and Lewis, 2001; Gnepp *et al.*, 2005). The diagnostic criteria for invasiveness degree of carcinoma

need to be further substantiated studied. Therefore, the use of a marker for malignant progression will provide a useful tool. Ki-67 is the most used proliferative marker, and it can be helpful for diagnosis, and determination of aggressiveness and prognosis of many cancers (Laitinen *et al*., 2008; Vacchi-Suzzi *et al.,* 2010; Aune *et al.,* 2011; Habberstad *et al.,* 2011; Kapur *et al*., 2011; Luporsi *et al.,* 2011; Seethala *et al.,* 2011; Tang *et al.,* 2011; Yang *et al.,* 2011).

The objective of the present work is to evaluate by Ki-67 expression the proliferative index of CXAP considering the phases of malignant transformation and histological subtypes.

MATERIAL AND METHODS

It was used 36 cases each of CXPA and PA, and 22 areas of PA in CXPA. All cases of CXPA were classified according to invasiveness (early and frankly invasive phase) and histopathological subtype. Ki-67 expression was detected by immunohistochemistry, using the clone M1B1 as the primary antibody (Immunotech, Marelille, France), EnVision plus (DAKO, 4001) as the detection system, and DAB (Sigma) as chromogen. Quantitation was made with the help of the IMAGELAB-2000® program, counting at least 1,000 cells of each case studied. Cells were considered positive when the nuclei were brown-stained. Data was statistically analysed using Wilcoxon's, Mann-Whitney and Kruskal-Wallis' nonparametric tests. Probability values < 0.05 were considered significant.

RESULTS

The PA group included 12 men and 24 women, with a mean age of 37.1 years, with 66.1% of the cases involving the parotid gland, followed by the minor salivary gland (22.2%) and submandibular gland (16.6%). The proportion of man and women in the CXPA group were similar, with a mean age of 57.5 years, and 75% of the cases occurring in the parotid gland, 13.9% in submandibular gland, and 11.1% in minor salivary gland. According to invasiveness 14 out of 36 cases were classified as early phase (no capsule invasion or minimally invasive, < 1.5mm), and 16 as frankly invasive (> 1.5mm). Salivary duct carcinoma was the most common subtype (15 cases, 41.7%), followed by

adenocarcinoma NOS (7 cases, 19.4%), myoepithelial carcinoma (7 cases, 19.4%), epithelial-myoepithelial carcinoma (5 cases, 13.9%) and squamous cell carcinoma and sarcomatoid carcinoma one case each (2.8%).

Ki-67 index for PA, residual areas of PA and CXPA were 6.7%, 6.9%, and 49.3% respectively **(Table 1).** Residual areas of the various CXPA subtypes showed similar Ki-67 index **(Table 2).** There were no statistical differences among the histological subtypes of CXPA. Higher values were found in sarcomatoid and squamous carcinomas, but they corresponded only to 1 case each **(Table 3)**. The values were higher in frankly invasive carcinomas in relation to early phase cases, but it was not statistically significant (44.3% x 52.5%) as shown on **Table 4.** Ki-67 is a useful marker in the differential diagnosis of PA and CXPA, even when in the early invasive phase **(Table 5).**

DISCUSSION

Carcinoma ex-pleomorphic adenoma is rare, presenting various subtypes and phases of invasiveness, leading to different biological behavior and prognosis. The concept of in-situ or intracapsular carcinoma in CXPA was introduced by LiVolsi and Perzin in 1977. Tortoledo et al. (1984) confirmed the prognostic significance of neoplastic extension beyond the capsule of CXPA using objective measurements. However regional metastatic dissemination from intracapsular CXPA and deaths from minimally invasive CXPA were reported (Lewis *et al*. 2001; Felix *et al*. 2002; Katabi *et al.,* 2010). It is well accepted that frankly invasive cases have a worse prognosis.

Diagnosis of early CXPA can be challenging, as the atypical cellular features and the area involved can be minimal, and criteria have not yet been fully established (Di Palma *et al*., 2005). Hypercellularity, capsule invasion, hyalinization, necrosis, cellular and nuclear atypia, and mitosis may be important atypical features that could indicate an increased risk to malignant change (Auclair and Ellis 1990; Takeda *et al.,* 1999). Due difficulties of diagnosis and to better understand malignant progression of CXPA, molecular and immunohistochemical studies have been performed. The p53 and c-erB-2 proteins seem to be involved in the early phases of malignant transformation of PA, and therefore they can be potentially useful for the diagnosis (Freitas *et al.* 2005).

Ki-67 is a reliable marker of cellular proliferation, that is the hallmark of malignant transformation and progression as it happens from PA to CXPA. In fact, various authors used Ki-67 to better understand the biology of CXPA and its value as a prognostic marker (Di Palma *et al.,* 2005; Katori *et al.,* 2007; Seethala *et al.,* 2011). In addition, Ki-67 index can be considered an useful tool in distinguishing PA from CXPA, because it is significantly higher in the carcinomatous areas (Freitas *et al.,* 2005).Our findings confirmed these observations, showing that CXPA present a higher proliferative index than PA and areas of residual PA in CXPA. On the other hand, there was no statistically significant difference between PA and residual PA. The latter showed higher values, and although it was not statistically significant, this indicates that it is at initial stages of malignant transformation.

The proliferative index showed no significant differences between the histological subtypes of CXPA, but in our series we had only intermediate and high-grade tumors. In fact sarcomatoid and squamous cell carcinoma showed very high values (75.4% and 81.1% of positive cells, respectively), but we had only one case each, precluding a definitive conclusion.

Frankly invasive CXPA showed proliferative index higher than those at early stages of invasiveness, but it was not statistically significant. In short Ki-67 seems to be useful to help to detect initial areas with malignant transformation, it does not help to better characterize the histological subtypes or aggressiveness that is better evaluated by histological invasiveness of the capsule and adjacent tissues.

Katori et al. (2007) also found a significant increase of Ki-67 index in CXPA, especially in adenocarcinoma NOS. However, there are controversies regarding to aggressiveness of each histopathological subtype (Katabi *et al*., 2010).

In conclusion, proliferative index determined by Ki-67 can be useful for differential diagnosis of PA and CXPA, even when in the early invasive phase, as the values in the carcinomatous areas are higher than in PA. On the other hand we did not find statistically significant difference of Ki67 index in the different subtypes of CXPA studied.

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TABLES

Table 1-Ki-67 index (%) in PA, areas of residual PA in CXPA and CXPA.

Cases	N	Min	Max	Mean ± SD	Mann-Whitney test	Mann-Whitney test	Wilcoxon test
Control PA	36	1.6	11.5	6.7 ± 2.5	Control PA	Control PA	Residual PA
Residual PA	22	0.6	30.2	6.9 ± 8.2	Residual PA	CXPA	CXPA
CXPA	36	11.6	86.2	49.3 ± 21.7	*p*=0.278	*p*=0.000	*p*=0.000

Table 2. Ki-67 mean index of histopathological subtypes between residual PA and CXPA.

	N	Mean ± SD (%)	Wilcoxon test
Adenocarcinoma NOS			
CXPA	7	59.3 ± 22.6	*p*=0.043
Residual PA	5	6.7 ± 10.1	
Duct salivary carcinoma			
CXPA	15	42.7 ± 18.8	*p*=0.012
Residual PA	8	4.1 ± 4.1	
Epithelial-myoepithelial carcinoma			
CXPA	5	44.3 ± 20.0	*p*=0.068
Residual PA	4	10.2 ± 13.4	
Myoepithelial carcinoma			
CXPA	7	48.6 ± 24.8	*p*=0.068
Residual PA	4	11.1 ± 6.8	

N=Number; SD=Standard deviation

Table 3- Histopathological subtypes of 36 CXPA and Ki-67 mean index in % of positive cells. Kruskal-Wallis test (p=0.294), showed no statistical differences among the subtypes.

Histopathological subtypes	**N (%)**	**Mean ± SD (%)**
Adenocarcinoma NOS	7 (19.4)	59.3 ± 22.6
Salivary duct carcinoma	15(41.7)	42.7 ± 18.8
Epithelial-myoepithelial carcinoma	5 (13.9)	44.3 ± 20.0
Myoepithelial carcinoma	7 (19.4)	48.6 ± 24.8
Sarcomatoid carcinoma	1 (2.8)	75.4 1*
Squamous cell carcinoma	1 (2.8)	81.1*
Total	**36 (100%)**	**49.3 ± 21.7**

*absolute number.

Table 4. Ki-67 mean index in 36 cases of early and frankly invasive CXPA.

Invasiveness	**N**	**Mean ± SD (%)**	**Kruskal Walis test**
Early invasive phase	14	44.3 ± 23.0	*p*=0.296
Franly invasive phase	22	52.5 ± 20.8	
Total	36	49.3 ± 21.7	

Table 5. Ki-67 mean index of early and frankly invasive CXPA compared to own residual PA.

Invasiveness	**N**	**Residual PA Mean ± SD (%)**	**N**	**CXPA Mean ± SD (%)**	**Kruskal Walis test**
Early invasive phase	**12**	6.4 ± 6.9	**14**	44.3 ± 23.0	*p*=0.002
Frankly invasive phase	**9**	4.9 ± 6.3	**22**	52.5 ± 20.8	*p*=0.008

CAPÍTULO 3

Artigo em preparação

ALTERATION OF COPIES NUMBER AND METHYLATION OF TUMOR SUPPRESSOR GENES INVOLVED IN CARCINOGENESIS OF CARCINOMA EX-PLEOMORPHIC ADENOMA

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ABSTRACT

INTRODUCTION: Carcinoma ex-pleomorphic adenoma (CXPA) arises from a benign pleomorphic adenoma (PA), and therefore it is an interesting model for to better understand the process of carcinogenesis. Some tumor suppressor genes are altered, presenting mutations, deletions and promoter methylation, but the mechanisms involved are multiple, complex and not yet well known. **OBJECTIVE:** The aim of the present study is to evaluate by Multiplex Ligation Dependent-Probe Amplification (MLPA) across a tumor suppressor genes panel, the characteristics of CXPA and PA, considering the histological subtypes and malignant progression. **MATERIAL AND METHODS:** Ten cases of CXPA, 10 cases of PA, and 5 areas of PA from CXPA were studied. All cases of CXPA were classified according to invasiveness (early and frankly invasive phase) and histopathological subtype. A genomic profile of copy number changes and in 41 different tumor suppressor genes by MLPA were studied in all the cases, using normal salivary gland tissue as control. Values lower than 0.7 were interpreted as losses, higher than 1.3 as gains and 2.5 or higher as amplifications. P-values <0.05 were considered significant. The methylation analysis was done by comparing the peak of the unmethylated probes in relation with methylated ones. **RESULTS:** CXPA group included 2 salivary duct carcinoma, 2 adenocarcinoma NOS, 4 epithelial-myoepithelial carcinoma and 1 sarcomatoid carcinoma. Four cases showed early and six frankly invasive phase. Alterations of *CASP8, MLH1*, and *RARB* genes were associated to PA tumorigenesis and CXPA malignant transformation. Areas de PA in CXPA showed more alterations than benign PA, loss of *KLK3* and methylation of *TIMP3, CDKN2A, ATM, HIC1, APC, MLH1, PTEN, BRCA2, RASSF1, DAPK1, TP73, BRCA1, CD44,* and *ESR1* genes. These alterations were also found in CXPA, particularly when frankly invasive. Silencing of *FHIT* and loss of *CTNNB1* genes were exclusive of frankly CXPAs, that also presented higher prevalence of alterations in *CDKNIB, VHL, CHFR, BRCA1, IGSF4, GSTP1*, *BCL2, CDH13,* and *TNFRSF7* genes. It was not found any correlation of genomic alterations and CXPA histopathological subtypes. **CONCLUSION:** These results indicate that various tumor suppressor genes are altered during PA tumorigenesis and CXPA carcinogenesis. Although benign PA and areas of PA in CXPA are morphologically similar, the latter shows genotypic changes not found in the first. With malignant transformation and invasiveness

of CXPA, there is an accumulative increase of genomic alterations that seems to be specific for each phase.

Key words: pleomorphic adenoma, carcinoma ex-pleomorphic adenoma, multiplex, loss of copies number, methylation, malignant progression.

INTRODUCTION

Carcinoma ex-pleomorphic adenoma (CXPA) is a rare malignant tumor, showing various histological subtypes and degree of invasiveness (Olsen and Lewis, 2001; Gnepp *et al.*, 2005). The CXPA arises from a pleomorphic adenoma (PA), and therefore it is an interesting model for the study of progression of an adenoma to carcinoma. CXPA carcinogenesis has been investigated by different methods, but the process is highly complex and remains unclear.

Tumor suppressor genes alterations, in particular *TP53* gene mutations, methylation, loss of chromosomal regions, deletions and overexpression were described in the pathogenesis of CXPA (Augello *et al.,* 2006; Fowler *et al*., 2006; Ihrler *et al.*, 2007). However, Gedlicka et al. (2010), found no *TP53* mutations, suggesting that the malignant progression of PA is *TP53* gene independent. Other tumor suppressor genes alterations have been described in the CXPA carcinogenesis process, including *P16, P21, RASSF1, RB, nm-23, DCC, hTERT, WT1, K-ras* (Yamamoto *et al*., 1996; Augello *et al*., 2006; Schache *et al*., 2010).

Methylation has been detected in a high percentage of tumors, and it is interesting target, because gene silencing by epigenetic changes is potentially reversible and therefore attractive for developing new therapeutic approaches (Miracca *et al*., 1999). Nevertheless there are few studies associated to methylation in CXPA, and tumor suppressor genes involved in its carcinogenesis deserve further investigations. Therefore, the objective of the present work is to determine the genomic profile of gain, loss and methylation of a panel of tumor suppressor genes, by Multiplex Ligation Probe-Dependent Amplification (MLPA) in PA and CXPA.

MATERIAL AND METHODS

Ten cases each of CXPA and PA, and 5 areas of residual PA in CXPA were used on this study. All 10 cases of CXPA were classified according to invasiveness (early and frankly invasive phase) and histopathological subtype.

Tumor DNA was extracted from paraffin embedded tissue obtained with the help of 3mm diameter punch, and using Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) in accord with the manufacturer's recommendations. To improve the quality of the isolated DNA, the protocol included steps especially used for paraffin embedded tissues, which includes deparaffination with xylene, followed by methanol washings and 24-hour incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet was dried and digested for 3 days in lysis buffer with high doses of proteinase K.

MLPA was performed as described previously, using the probe mixture ''SALSA MS-MLPA ME001-C1 tumor suppressor probemix" (MRC-Holland, Amsterdam, the Netherlands; www.mlpa.com). Genes, chromosomal regions, and probe sequences are listed in **Table 1**. This mix enabled the analysis of 41 genes that according to the literature may be involved in carcinogenesis (Worsham *et al.,* 2006; Henken *et al.*, 2007). Each probe is composed of two parts that hybridize to adjacent target sequences in the DNA. After the ligation step and PCR amplification, each probe gives rise to a product with a unique size between 130 and 480 nt. Briefly, 100 ng DNA was denatured at 98° C for 5 min and hybridized with the MLPA probe mixture at 60° C for 16 h. Ligation of the two parts of each probe was performed by a thermostable ligase. All probe ligation products include a same sequences and were amplified by PCR using the same primer pair at 60° C for 1 min, 33 cycles of 95°C 30s, 60°C 30s and 72°C for 1 min, followed by 20 min at 72°C and kept cold at 4°C.

The MLPA probes for quantification of methylation are similar to normal MLPA probes for detecting the number of copies, except that the sequence detected by probe methylation was recognized by the restriction enzyme sensitive to methylation (Hhal). Twenty four of the studied genes have promoter region for methylation. The reaction was performed in two tubes, one was processed only with the hybridization reaction described above, which will provide information about changes in the number of copies. In the other

tube, hybridization reaction occurred concomitantly with the digestion of unmethylated probes, incubated with the enzyme Hhal. The probes not digested were amplified by PCR, and analyzed by capillary electrophoresis (Sellnor and Taylor, 2004). All the products (copies number and methylation) were subsequently analyzed on an ABI Prism 3100 sequencer and by Peak Scanner v1.0 software (Applied Biosystems, Warrington, UK).

Normal DNA from four different normal salivary glands were used as reference to calculate median values and standard deviations for every probe. Each tumor sample was analyzed at least twice. For every gene, the relative copy number was calculated by dividing the average relative peak area of the tumor by the median relative peak area of the normal reference samples. A normal DNA resulted in relative copy numbers varying between 0.85 and 1.15, for each probe. Therefore, relative copy number values, considering the standard deviation, lower than 0.7 were interpreted as losses, higher than 1.3 as gains and 2.5 or higher as amplifications. This interpretation of MLPA data is in accord with Moerland et al. (2006) who found a 98% concordance with data obtained by FISH spot counting, both performed on paraffin tumor material. P-values <0.05 were considered significant. The methylation analysis was done by comparing the peak of the unmethylated probes with methylated ones.

RESULTS

Demographic data of the 10 cases each of PA and CXPA are shown on **Table 2.** Four cases of CXPA showed early phase of invasion (intracapsular or <1.5mm of invasion), and 6 cases were frankly invasive (>1.5 mm). Histologically CXPA were subtypes as 2 cases of salivary duct carcinoma (SDC), 2 adenocarcinoma not otherwise specification (AdNOS), 4 epithelial-myoepithelial carcinoma (EMC) and 1 sarcomatoid carcinoma (SC). The demographic data of PA and CXPA cases are summarized in **Table 2.**

The most common genes with loss of copies number in PA were *RARB* and *CASP8* (8/10) followed by *MLH1*gene (7/10). Other loss of copies number and methylated genes are found in **Table 3**.

Residual areas of PA in CXPA presented an increased loss of copies number and methylated genes when compared to PA cases, besides similar alterations found in *RARB,*

CASP8, and *MLH1* in PA as cited above. The *KLK3* gene showed loss of copies number (3/5), and various other genes showed a high index of methylation, as *TIMP3, CDKN2A, ATM, HIC1* (5/5); *PTEN, BRCA2, RASSF1, DAPK1, TP73* (4/5 - 80%) and *BRCA1, CD44, ESR1* (3/5). The genes *MLH3, PAH, CHFR, BCL2, CDKN1B, VHL, AI651963, BRCA2, IGSF4, CDH13, TNFRSF7, GSTP1* also showed alterations in residual PAs, and the differences in the genomic profile between PA and residual areas of PA in CXPA are summarized in **Table 3.**

The *CASP8* gene again was the most involved with loss of copies number in the CXPA group (7/10). The *BCL2* and *KLK3* genes showed loss in 6/10 cases. On the other hand, the *TIMP3* gene was methylated in all ten cases of CXPA, similar to residual PA cases. Other genes were methylated in a significant number of CXPA: *PTEN* and *ATM* (9/10), *CDKN2A* and *HICI* (8/10). Other findings are shown in **Table4**.

Considering the invasiveness degree, early invasive CXPA showed loss of copies number of *CASP8* (3/4) and methylation of *TIMP3* in all four cases. *CDKN2A, ATM, PTEN* genes also were methylated in 3/4 cases. The frankly invasive CXPAs showed more alterations when compared to PA, residual areas of PAs and early invasive CXPA. *BCL2* and *CDH13* genes presented loss of copies number in 5/6 cases. *TIMP3, APC, ATM, RARB, HIC1, CHFR, BRCA1*, and *PTEN* genes were methylated in all six cases and the *CDKN2A, CDKN1B, BRCA2, MLH1, RASSF1, TP73* genes were methylated in 5/6 cases. Methylation was found also in the *DAPK1, VHL, ESR1, IGSF4* genes (4/6). Two genes showed alterations only in frankly invasive cases, as loss of copies of *FHIT* (3/6) and methylation of *CTNNB1 (2/6).* The genomic panel of frankly and early invasive CXPAs is summarized in the **Table 4**.

DISCUSSION

Carcinoma ex-pleomorphic adenoma is an interesting model of carcinomatous progression, as it is derives from a benign counterpart usually present in the tumor (Gnepp *et al*., 2005). Also, it presents various subtypes, classified as high, medium and low grade carcinomas, as SDC, undifferentiated carcinoma (UC), polymorphous low grade

adenocarcinoma (PLGA), MC, EMC, SC, epidermoid carcinoma (EC), adenoid cystic carcinoma (ACC), clear cell carcinoma (CCC), mucoepidermoid carcinoma (MC), basal cell carcinoma (BCC), oncocytic carcinoma (OC), and AdNOS. The most frequent are poorly differentiated adenocarcinomas, SDC, or AdNOS (Olsen and Lewis, 2001; Gnepp *et al.,* 2005). In our sample of 10 cases, there were 4 cases of EMC, 2 SDC, 2 AdNOS, 1 MC and 1 SC, and of these, 4 were early and six frankly invasive.

It is estimated that about 1.6 - 7.5% of untreated or recurrent PA undergo malignant changes, and the risk increases with time of disease progression (Eneroth 1964; Eneroth *et al*., 1968). The pathogenesis of the malignant transformation of PA to CXPA is still poorly understood, although several genetic and molecular changes have already been described.

Malignant transformation is a multistep process, with activation of oncogenes and inactivation of tumor suppressor genes (Vogelstein *et al*., 2004). The tumor suppressor gene *TP53* has been shown have a role in the development and progression of CXPA, presenting mutations, deletions and overexpression (Fowler *et al*., 2006; Ihrler *et al.*, 2007). Other tumor suppressor genes also have been shown to be altered in CXPA as *P16, P21, RASSF1, RB, nm-23, DCC, hTERT, WT1 and K-ras* (Yamamoto *et al*., 1996; Augello *et al*., 2006; Schache *et al*., 2010). Nevertheless these alterations must be better understood, and possibly other genes may also be involved.

The current study shows alterations of several tumor suppressor genes not yet described in CXPA carcinogenesis and PA tumorigenesis. The loss of copies of *CASP8* was observed in PA, areas of PA in CXPA, up to frankly invasive CXPA, showing that it is associated with PA tumorigenesis and malignant transformation, and CXPA progression to high degree of invasiveness. The *CASP8* (caspase 8, apoptosis-related cysteine peptidase) located at 2q33.2 has a central role in the execution-phase of cell apoptosis (Kaneda *et al.;* 2006; Liu *et al*., 2011). Alterations of *CASP8* in PA and CXPA had not been previously described.

The highest loss of *MLH1* was found in PA (70%). In the later phases, a decrease of loss of *MLH1* was observed: residual PA (60%), early CXPA (25%) and frankly CXPA (33.3%). Nevertheless, methylation of this gene was observed only in residual areas of PA and CXPAs, but not in PA. So, inactivation of *MLH1* may contribute for progression from

PA to CXPA, whereas the function decrease is mainly important for PA tumorigenesis. The *MLH1* (MutL homolog 1) gene is located on chromosome 3 (3p22.10) and encodes proteins involved in the repair mismatches of DNA. The deficiency or defect of *MLH1* gene associated to pathogenesis of benign and malignant tumors were already described (Ramírez-Ramírez *et al.,* 2011; Wang *et al*., 2011). On the other hand, in two cases of residual PA and one of frankly invasive CXPA, it was observed a gain of *MLH1* gene, and this could be interpreted as a form of protection.

Similarly, loss of *RARB* (3p24) decreased with progression from PA to early CXPA, and frankly invasive CXPA showed normal levels of *RARB* gene. Nevertheless the methylation rate of *RARB* increased from residual PA to frankly CXPA, with no alterations in PA, suggesting that its inactivated function can be relevant for CXPA carcinogenesis. The methylation and loss of this gene has not been described in PA and CXPA, however it is frequently inactivated in cancers of epithelial origin (Pappas *et al*., 2011; Schache *et al.,* 2010).

It is interesting that various genes showed increased alterations when PA and areas of residual PA in CXPA were compared, although morphologically they are similar. These included loss of copies of *KLK3,* and important rate of methylation of *TIMP3, CDKN2A, ATM, HIC1, APC, MLH1, PTEN, BRCA2, RASSF1, DAPK1, TP73, BRCA1, CD44, ESR1* genes. Most of these changes were maintained in early and frankly invasive CXPA cases, except silencing in early CXPA of *BRCA1, BRCA2, DAPK1, CD44*, and *ESR1* genes. Other eleven genes (*MLH3, PAH, CHFR, BCL2, CDKN1B, VHL, AI651963, IGSF4, CDH13, TNFRSF7, GSTP1)* also showed alterations in residual areas of PA, and in CXPA, but not in PA. Therefore, most of the genomic changes acquired in residual PA are maintained and/or increased until the most aggressive phase of CXPA.

Genetic alterations are found when benign and malignant areas of the same CXPA are compared. Usually there is an increase in copy loss or methylation, but it is not so in all cases. Two areas of residual PAs (from one frankly and one early CXPA cases) showed an increased of loss and methylated genes when compared to carcinomatous regions, suggesting that the malignant progression may occur from different cellular clones.

Early invasive CXPA showed less genetic alterations than frankly invasive cases, and this may be associated to a more indolent clinical behavior of these CXPA (Gnepp *et al*., 2005). It should be emphasized that silencing of *FHIT* and loss of *CTNNB1* genes were exclusive findings in frankly CXPAs. The methylation of *CDKNIB, VHL, CHFR, BRCA1, IGSF4, GSTP1*, and loss of copies number of *BCL2, CDH13, TNFRSF7* genes also had highest prevalence in frankly invasive CXPA.

One case of CXPA and two residual areas of PA in CXPA, showed a different genomic profile compared with the other cases, showing that as expected the process is not uniform, with a few cases presenting unique molecular changes. Regarding histopathological characterisitics, we did not find a genetic pattern for each subtype included on this study.

In summary, CXPA is an interesting model for the study of benign-malignant progression, because it arises in or from a benign PA. This study shows that various tumor suppressor genes are altered in CXPA (mainly *TIMP3, APC, CDKN2A, MLH1, ATM, RARB, HIC1, CHFR, BRCA1, CDKN1B, CASP8, PTEN, BRCA2, DAPK1, AI651963, ESR1, KLK3, FHIT, IGSF4, CDH13, RASSF1, TP73*), and that some of these alterations can be found in a lower intensity in areas of PA in CXPA (*RARB, CHFR, BRCA1, CDKN1B, AI651963, IGSF4, CDH13*) and also in PA (*TIMP3, APC, CDKN2A, ATM, HIC1, CHFR, BRCA1, CDKN1B, PTEN, BRCA2, RASSF1, DAPK1, AI651963, ESR1, KLK3, TP73, FHIT, IGSF4, CDH13*). Some alterations were found only in the malignant areas of CXPA (*CTNNB1, FHIT*).

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TABLES

Table 1 – Tumor suppressor probemix used in current study.

GENES	SITE	PROBES SEQUENCES
CREM	10p12.1	GCTCCTCCACCAGGTGCTACAAT - TGTACAGTACGCAGCACAATCAGCTGATGGCACACAGCAGT
TIMP3+	22q12.3	TCCA**GCGC**CGAGGCAGCCTCGC - T**GCGC**CCCATCCCGTCCCGCCGGGCACTCGG
APC+	5q22	CAGCTGTGTAATCCGCTGGATGCGGACC - AGG**GCGC**TCCCCATTCCCGTCGGGAGCCCGC
PARK2	6q26	CGTTCACGACCCTCAACTTGGCTACT - CCCTGCCTTGTGTGGGTAAGTCTAGCATGTTTTCTCTCCAT
CDKN2A+	9p21	CAGAGGGGAAGAGGAAAGAGGAAGAA**GCGC**TCAGAT - GCTCCGCGGCTGTCGTGAAGGTTAAAACCGAAAATAAAAATGG
MLH1+	3p22.1	CGTTGAGCATCTAGACGTTTCCTTGGCTCT - TCTG**GCGC**CAAAATGTCGTTCGTGGCAGGGGTTATTC
TNFRSF1A	12p13	TGCCACACTGCCCTGAGCCCAA - ATGGGGGAGTGAGAGGCCATAGCTGTCTGGC
ATM+	11q23	GGAGGGAGGAGGCGAGAGGAGTCGGGA - TCT**GCGC**TGCAGCCACCGCCGCGGTTGATACTACTTT
RARB+	3p24	CCGCCGGCTTGT**GCGC**TCGCT - GCCTGCCTCTCTGGCTGTCTGCTTTTGCAGGGCTGCT
MLH3	14q24.3	GCGACCTTGTTCTTCCTTTCCTTCCGA - GAGCTCGAGCAGAGAGGACTGTGATGAGACAGGATAACAG
CDKN2B+	9p21	CTGCGACAGCTCCTGGAAGCCGG - **CGCG**GATCCCAACGGAGTCAACCGTTTCGGGAGG
HIC1+	17p13.3	CCGCTCCAGATAAGAGTGTGCGGA - AA**GCGC**GGCGGGGCTGAGACGCGACCAGGAC
PAH	12q23	CAGTGCCCTGGTTCCCAAGAA - CCATTCAAGAGCTGGACAGATTTGCCAATCAGATTCTCAG
CHFR+	12q24.33	CGCGAGAGTAG**GCGC**GTGGAGG - A**GCGC**TCGGCCATCTTTGATCCTGACCAGGCGACTTCGT
BRCA1+	17q21	TTCTCAGATAACTGGGCCCCT**GC** - **GC**TCAGGAGGCCTTCACCCTCTGCTCTGGGTAAAGGT
BCL2	18q21.3	CTTCTCCTGGCTGTCTCTGAAGACTC - TGCTCAGTTTGGCCCTGGTGGGAGCTTGCATC
CASP8+	2q33.2	CTTTCCAATAAAGCATGTCCA**GCGC**TC - GGGCTTTAGTTTGCACGTCCATGAATTGTCTGCCACA
CDKN1B+	12p13.2	AGCCCCT**GCGCGC**TCCTAGA - GCTCGGGCCGTGGCTCGTCGGGGTCTGTGTCTTT
TSC2	16p13.3	GAGCCAGAGAGAGGCTCTGAGAAGAAG - ACCAGCGGCCCCCTTTCTCCTCCCACAGGGCCTCCTG
PTEN+	10q23.3	CACCGGAGCGG**GCGC**AGGAGA - GGCCTGCGGGGTGCGTCCCACTCACAGGGAT
BRCA2+	13q12.3	CGGGAGAAGCGTGAGGGGACAGATTTGTG - CCG**GCGC**GGTTTTTGTCAGCTTACTCCGGCCAAAAAAGA
CDK6	7q21.3	GCGTGATTGGACTCCCAGGAGAAGAAGACT - GGCCTAGAGATGTTGCCCTTCCCAGGCAGGCTTTTCA
CD44+	11p12	CTCCTTTCGCCC**GCGC**CCTCC - GTTCGCTCCGGACACCATGGACAAGTTTTGGTGG
RASSF1+	3p21.3	CAGTCCCTGCACCCAGGTTTCCA - TT**GCGC**GGCTCTCCTCAGCTCCTTCCCGCCGC
CDH1	16q22.1	CTATGAAGGAAGCGGTTCCGAAGCTGCTA - GTCTGAGCTCCCTGAACTCCTCAGAGTCAGACAAAGACCAGGAC
DAPK1+	9q22	CGCGAGGATCTGGAGCGAACTGCT - **GCGC**CTCGGTGGGCCGCTCCCTTCCCTCCCT
VHL+	3p25.3	GCGAAGACTACGGAGGTCGACTCGGG - A**GCGCGC**ACGCAGCTCCGCCCCGCGTCCGACC
AI651963	10p14	CAATTGCCATTTTTTCCTGACATTCACTGT - GGAAATTTGGTGCACGACACTGTTAGGGGAGATCTGT
ESR1+	6q25.1	CGCCCGCCGTGTACAACTACCCCG - AGG**GCGC**CGCCTACGAGTTCAACGCCGCGGC
RASSF1+	3p21.3	GTCCACAGGGCGGGCCCCGAC - TTCA**GCGC**CTCCCCCAGGATCCAGACTG
KLK3	19q13	TGTGTCACCATGTGGGTCCCG - GTTGTCTTCCTCACCCTGTCCGTGACGTGGA
TP73+	1p36	CGCCCGCGAAGGGGACGCAGC - GAAACCGGGGCCC**GCGC**CAGGCCAGCCGGGA
FHIT+	3p14.2	CGCGGGTCTGGGTTTCCACGC - **GCG**TCAGGTCATCACCCCGGAGCCCAGTGGG
BRCA2	13q12.3	GGCCATGGAATCTGCTGAACAAAA – GGAACAAGGTTTATCAAGGGATGTCACAACCGTGTGGAAGTTGCGT
IGSF4+	11q23	CCTGGAGCCCGAGTCCTTGCACGCCA - G**GCGC**CCGGGAGAACACTTTTTCCTTGATCCGGGGAAAGCA
CDH13+	16q24.2	GTTCTGTGCGTTCTCCTGTCCCAG - GTAGGGAAGAGGGGCTGCCGG**GCGCGC**TCTG
TNFRSF7	12p13	GAAAGTCCTGTGGAGCCTGCA - GAGCCTTGTCGTTACAGCTGCCCCAGGGAGG
GSTP1+	11q13	CGAAGAGCGGCCG**GCGC**CGTG - ACTCAGCACTGGGGCGGAGCGGGGCGGGACC
MLH1+	3p22.1	CTGCTGAGGTGATCTG**GCGC**AGA - GCGGAGGAGGTGCTTG**GCGC**TTCTCAGGCTCCTCCTCT
CTNNB1	3p22	GGCTGTTAGTCACTGGCAGCAACA - GTCTTACCTGGACTCTGGAATCCATTCTGGTGCCACT
CASR	3q21	CCAGTGCCTGTAACAAGTGCCCAGATGACT - TCTGGTCCAATGAGAACCACACCTCCTGCATTGCCAAGGA

The Hha1 sites are marked with grey and in bold. + genes with site for Hha1.

Table 2-Demographic data 10 cases each of PA and CXPA cases used on this study**.**

	PLEOMORPHIC ADENOMA									
VARIABLE	**PA-1**	**PA-2**	**PA-3**	**PA-4**	**PA-5**	**PA-6**	**PA-7**	**PA-8**	**PA-9**	**PA-10**
Age	80	36	64	41	13	9	61	42	46	23 anos
Gender	Male	Male	Female	Male	Female	Male	Male	Female	Female	Female
Duration time	5 years	2 years	2 years	10 years	12 months	8 months	3 years	6 months	2 years	2 months
Site	Palate	Palate	Parotid	Parotid	Parotid	Parotid	Parotid	Parotid	Parotid	Parotid
Size (cm)	3.4	1.5	3	3	3	4	4	1.5	1	3
Treatment	S	S	S	S	S	S	S	S	S	S
Current time	DR	AWD	AWD	AWD	AWD	AWD	AWD	AWD	AWD	AWD
Follow up (months)	5	10	52	70	57	16	2	60	33	20
	CARCINOMA EX-PELOMORPHIC ADENOMA									
VARIABLE	**CXAP-1**	**CXPA-2**	**CXAP-3**	**CXAP-4**	**CXAP-5**	**CXAP-6**	**CXAP-7**	**CXAP-8**	**CXAP-9**	**CXAP-10**
Age	61	69	30	64	82	64	56	54	65	50
Gender	Female	Male	Female	Male	Female	Male	Male	Male	Male	Female
Duration time	20 years	4 months	2 years		20 years	20 years	5 years	4 years	2 years	20 years
Site	Parotid	Upper lip	Parotid	Parotid	Parotid	Parotid	Palate	Parotid	Parotid	Parotid
Size (cm)	5	2	7.5		6.2	4		2.8	8	8
Estadio	III	I	III		IVa	II		III	IVa	III
Subtype	MC	AdNOS	EMC	SDC	SC	EMC	EMC	AdNOS	SDC	EMC
Invasiveness	EI	EI	FI	FI	FI	FI	FI	FI	FI	FI
Treatment	S + RT	S	S	S	S + RT	S + RT	S	S	S	S
Current time	AWD	AWD	-	AWD	AWD	DR	DOD	AWD	AWD	-
Follow up (months)	36	27	-	12	24	45	48	12	6	-

PA=Pleomorphic adenoma; S=Surgery; DR= Dead for other reasons; AWD=Alive without disease; CXPA=Carcinoma ex-pleomorphic adenoma; MC=Myoepithelial carcinoma; AdNOS=Adenocarcinoma not otherwise specified; EMC=Epithelial-myoepithelial carcinoma; SDC=Salivary duct carcinoma; SC=Sarcomatoid carcinoma; EI=Early invasiveness; FI=Frankly invasiveness; RT=Radiotherapy; DOD=Dead of disease.

Table 3- Genomic profile of PA and residual PAs in CXPA cases.

		PLEOMORPHIC ADENOMA										RESIDUAL PLEOMORPHIC ADENOMA				
GENES	**SITE**	**PA-1**	**PA-2**	**PA-3**	**PA-4**	**PA-5**	**PA-6**	**PA-7**	**PA-8**	**PA-9**	**PA-10**	**RPA-1**	**RPA-2**	**RPA-3**	**3A3A-AP**	**1A1A-AP**
CREM	10p12.1		loss										loss			
TIMP3	22q12.3				loss							***methyl***	***methyl***	***methyl***	***methyl***	***methyl***
APC	5q22			methyl								***methyl***	***loss/methyl***		***methyl***	***methyl***
PARK2	6q26									loss			loss		loss	
CDKN2A+	9p21			methyl								***methyl***	***methyl***	***methyl***	***methyl***	***methyl***
MLH1	3p22.1	loss			loss		loss	loss	loss	loss	loss	gain/methyl	loss	loss/methyl	gain/methyl	loss/methyl
TNFRSF1A	12p13															
ATM	11q23			methyl								***methyl***	***methyl***	***methyl***	***methyl***	***methyl***
RARB	3p24	***loss***	***loss***		***loss***		***loss***	***loss***	***loss***	***loss***	***loss***		loss	methyl	methyl	loss
MLH3	14q24.3											loss	loss			
CDKN2B	9p21															
HIC1	17p13.3											***methyl***	***methyl***	***methyl***	***methyl***	***methyl***
PAH	12q23												loss			
CHFR	12q24.33														methyl	methyl
BRCA1	17q21												***methyl***		***methyl***	***methyl***
BCL2	18q21.3													loss	loss	
CASP8	2q33.2	loss		loss	loss		loss	loss	loss	loss	loss	loss	loss	loss	loss	loss
CDKN1B	12p13.2												methyl			
TSC2	16p13.3															
PTEN	10q23.3												***methyl***	***methyl***	***methyl***	***methyl***
BRCA2	13q12.3												***methyl***	***methyl***	***methyl***	***methyl***
CDK6	7q21.3															
CD44	11p12												***methyl***		***methyl***	***methyl***
RASSF1	3p21.3												***methyl***	***methyl***	***methyl***	***methyl***
CDH1	16q22.1															
DAPK1	9q22												***methyl***	***methyl***	***methyl***	***methyl***
VHL	3p25.3														methyl	
AI651963	10p14												loss			
ESR1	6q25.1												***methyl***	***methyl***		***methyl***
RASSF1	3p21.3			methyl	methyl			methyl					methyl	methyl	loss/methyl	methyl
KLK3	19q13												***loss***	***loss***	***loss***	
TP73	1p36											***loss***	***methyl***	***methyl***	***loss/methyl***	***methyl***
FHIT	3p14.2															
BRCA2	13q12.3												loss			
IGSF4	11q23														methyl	
CDH13	16q24.2													loss	loss	methyl
TNFRSF7	12p13													loss		
GSTP1	11q13															methyl
MLH1	3p22.1															
CTNNB1	3p22															
CASR	3q21															

The differences in findings between PA and residual PA are in bold and underlined. PA=Pleomorphic adenoma; RPA = Residual pleomorphic adenoma.

Table 4-Genomic profile of early and frankly invasive CXPAs.

		EARLY INVASIVE CARCINOMA EX-PLEOMORPHIC ADENOMA						FRANKLY INVASIVE CARCINOMA EX-PLEOMORPHIC ADENOMA								
GENES	**SITE**	**RPA-1**	**CXAP-1**	**CXAP-2**	**RPA-2**	**CXAP-3**	**CXAP-4**	**CXAP-5**	**CXAP-6**	**RPA-3**	**CXAP-7**	**RPA-4**	**CXAP-8**	**CXAP-9**	**RPA-5**	**CXAP-10**
CREM	10p12.1				*loss*			*loss*								
TIMP3	22q12.3	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl
APC	5q22	methyl		methyl	loss/methyl			loss/methyl	loss/methyl		methyl	methyl	methyl	methyl	methyl	methyl
PARK2	6q26				*loss*			loss				loss				
CDKN2A	9p21	methyl	methyl	methyl	methyl	methyl		loss/methyl	methyl	methyl	methyl	methyl	methyl		methyl	methyl
MLH1	3p22.1	gain/methyl		methyl	loss	loss		loss/methyl	loss/methyl	loss/methyl	methyl	gain/methyl	gain/methyl	methyl	loss/methyl	methyl
TNFRSF1A	12p13															
ATM	11q23	methyl	methyl	methyl	methyl	methyl		methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl
RARB	3p24		loss	methyl	loss			methyl	methyl	methyl	methyl	methyl	methyl	methyl	loss	methyl
MLH3	14q24.3	loss	loss		loss			*loss*								
CDKN2B	9p21							loss								
HIC1	17p13.3	methyl	methyl	methyl	methyl			methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl
PAH	12q23				loss	loss		*loss*								
CHFR	12q24.33			*methyl*				methyl	methyl		methyl	methyl	methyl	methyl	methyl	methyl
BRCA1	17q21				*methy*l			methyl	methyl		methyl	methyl	methyl	methyl	methyl	methyl
BCL2	18q21.3			*loss*				loss	loss	loss	loss	loss	loss	loss		
CASP8	2q33.2	loss		loss	loss	loss	loss	methyl	loss	loss	loss	loss	loss	loss	loss	
CDKN1B	12p13.2				*methyl*			methyl	methyl		methyl		methyl	methyl		
TSC2	16p13.3															
PTEN	10q23.3		methyl	methyl	methyl	methyl		methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl
BRCA2	13q12.3				*methyl*			methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	
CDK6	7q21.3															
CD44	11p12			loss	methyl			loss/methyl	methyl		methyl	methyl	loss		methyl	
RASSF1	3p21.3		methyl		methyl	methyl		methyl	methyl	methyl	methyl	methyl	methyl	methyl	methyl	
CDH1	16q22.1			*loss*												
DAPK1	9q22			loss	methyl			methyl	methyl	methyl	methyl	methyl	methyl		methyl	
VHL	3p25.3							methyl	methyl		methyl	methyl		methyl		
AI651963	10p14				loss	loss	loss	loss			loss			loss		
ESR1	6q25.1			loss	methyl			methyl	methyl	methyl	methyl		methyl		methyl	
RASSF1	3p21.3			loss	methyl	methyl		methyl	methyl	methyl	methyl	loss/methyl	loss/methyl	methyl	methyl	
KLK3	19q13				loss	loss	loss	loss		loss	loss	loss	loss	loss		
TP73	1p36	loss		loss	methyl	methyl		methyl	methyl	methyl	methyl	loss/methyl	loss/methyl	methyl	methyl	
FHIT	3p14.2							methyl	methyl		methyl					
BRCA2	13q12.3				*loss*											
IGSF4	11q23							methyl	methyl		methyl	methyl	methyl			
CDH13	16q24.2							loss/methyl	loss	loss	loss	loss	loss	loss	methyl	
TNFRSF7	12p13									loss	loss		loss	loss		
GSTP1	11q13							methyl			methyl			methyl	methyl	
MLH1	3p22.1												loss			
CTNNB1	3p22										loss		loss			
CASR	3q21															

RPA=Residual pleomorphic adenoma; CXPA=Carcinoma ex-pleomorphic adenoma. The cases RPA-1 and CXPA-1 are benign and malignant area respectively, and belong to the same tumor. RPA-2 and CXPA-3, RPA-3 and CXPA-7, RPA-4 and CXPA-8, RPA-5 and CXPA-10.

Table 5- Summary of findings.

		PA - 10 CASES		RESIDUAL PA - 5 CASES			EARLY CXPA - 4 CASES		FRANKLY CXPA - 6 CASES		
GENES	**SITE**	**LOSS N(%)**	**METHYL N(%)**	**GAIN N(%)**	**LOSS N(%)**	**METHYL N(%)**	**LOSS N(%)**	**METHYL N(%)**	**GAIN N(%)**	**LOSS N(%)**	**METHYL N(%)**
CREM	10p12.1	1(10)			1(20)					1(16.6)	
TIMP3	22q12.3	1(10)				5(100)		4(100)			6(100)
APC	5q22		1(10)		1(20)	4(80)		1(25)		2(33.3)	6(100)
PARK2	6q26	1(10)			2(40)					1(16.6)	
CDKN2A	9p21		1(10)			5(100)		3(75)		1(16.6)	5(83.3)
MLH1	3p22.1	7(70)		2(40)	5(100)	4(80)	1(25)	1(25)	1 (16.6)	3(50)	5(83.3)
TNFRSF1A	12p13										
ATM	11q23		1(10)			5(100)		3(75)			6(100)
RARB	3p24	8(80)			2(40)	2(40)	1(25)	1(25)			6(100)
MLH3	14q24.3				2(40)		1(25)			1(16.6)	
CDKN2B	9p21									1(16.6)	
HIC1	17p13.3					5(100)		2(50)			6(100)
PAH	12q23				1(20)		1(25)			1(16.6)	
CHFR	12q24.33					2(40)		1(25)			6(100)
BRCA1	17q21					3(60)					6(100)
BCL2	18q21.3				2(40)		1(25)			5(83.3)	
CASP8	2q33.2	8(80)			5(100)		3(75)			4(66.6)	1(16.6)
CDKN1B	12p13.2					1(20)					5(83.3)
TSC2	16p13.3										
PTEN	10q23.3					4(80)		3(75)			6(100)
BRCA2	13q12.3					4(80)					5(83.3)
CDK6	7q21.3										
CD44	11p12				3(60)	3(60)	1(75)			2(33.3)	2(33.3)
RASSF1	3p21.3					4(80)		2(50)			5(83.3)
CDH1	16q22.1						1(25)				
DAPK1	9q22					4(80)	1(25)				4(66.6)
VHL	3p25.3					1(20)					4(66.6)
AI651963	10p14				1(20)		2(50)			3(50)	
ESR1	6q25.1					3(60)	1(25)				4(66.6)
RASSF1	3p21.3		3(30)		1(20)	4(80)	1(25)	1(25)		1(16.6)	5(83.3)
KLK3	19q13				3(60)		2(50)			4(66.6)	
TP73	1p36				2(40)	4(80)	1(25)	1(25)		1(16.6)	5(83.3)
FHIT	3p14.2										3(49.9)
BRCA2	13q12.3				1(20)					1(16.6)	
IGSF4	11q23					1(20)					4(66.6)
CDH13	16q24.2				2(40)	1(20)				5(83.3)	1(16.6)
TNFRSF7	12p13				1(20)					3(50)	
GSTP1	11q13					1(20)					3(50)
MLH1	3p22.1									1(16.6)	
CTNNB1	3p22									2(33.3)	
CASR	3q21										

CAPÍTULO 4

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CARCINOMA EX-PLEOMORPHIC ADENOMA OF UPPER LIP SHOWING COPIES NUMBER LOSS OF TUMOR SUPRESSOR GENES

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ABSTRACT

Carcinoma ex-pleomorphic adenoma (CXPA) is a malignant salivary gland tumor that arises mainly in the parotid gland, and rarely in the minor salivary glands. The main site of CXPA in the minor salivary glands is the palate, but the involvement of other regions has also been described in the literature. The etiology of CXPA remains unclear and different molecular biology studies have been made to explain the carcinogenesis of the tumor. The role of some tumor suppressor genes in CXPA is well known, but other types still need to be studied. **OBJECTIVE:** We present a uncommon case of CXPA involving the upper lip together with panel of tumor suppressor genes by Multiplex Ligation Dependent-Probe Amplification (MLPA). **RESULTS:** A 69-year-old male was submitted to surgical resection of a mass in the mucous of the upper lip histologically diagnosed as CXPA. The patient is free of disease after 30 months postoperatively. From all genes investigated in this study, we detected loss of copies number of *BCL2, CASP8, CD44, CDH1, DAPK1, ESR1, RASSF1*, and *TP73*. **CONCLUSIONS:** CXPA is rare in mouth, but it should be considered in the differential diagnosis of minor salivary gland malignancies and the genomic findings this case may be an important tool in understanding the carcinogenesis process of CXPAs.

Key words: Carcinoma ex-pleomorphic adenoma, minor salivary gland, multiplex, tumor suppressor genes, upper lip.

INTRODUCTION

Carcinoma ex-pleomorphic adenoma (CXPA) is a rare malignant tumor that affects the salivary glands, corresponding to 3.6% of all salivary neoplasms and 11.7% of all salivary malignancies[1,2]. The CXPA occurs more commonly in the parotid and submandibular glands, being extremely rare in minor salivary glands[3]. The main location of CXPA of the minor salivary gland is the palate, but other locations were described such as buccal mucosa, floor of the mouth, alveolar ridge, gingiva, retromolar area, nasopharynx, and upper and lower lips[4-12].

The pleomorphic adenoma (PA) malignant transformation process remains unclear. The hypothesis is that development and behavior of tumor cells is determined by genetic changes in essential biological events. So, the study of control cellular cycle control over tumor suppressor genes offers clues to the tumorigenesis process.

A limited group of genes and markers of PA malignant progression was described in previous studies[13-15]. Therefore, the objective of this work is to contribute with the investigation of CXPA carcinogenesis showing copy number loss of a different panel of tumor suppressor genes by Multiplex Ligation Probe Dependent Amplification (MLPA) and to report a rare case of CXPA involving the upper lip.

PATIENT AND METHODS

A 69-year-old man was referred to Oral Diagnosis Service for evaluation of a nodule on the right upper lip. The patient reported that he first noticed the nodule 4 years ago. On clinical examination, it was observed a nodular painless lesion on the right side of the upper lip, with fibroelastic consistency, measuring approximately 2.0 x 2.0 cm **(Fig. 1).** Pleomorphic and canalicular adenoma were the main clinical diagnostic hypothesis and so it was surgically removed.

Microscopical examination of the surgical specimen showed an uncapsulated well-circumscribed lesion, showing intense hyalinization with clusters and nests of epithelial cells, and ductal structures composed of epithelial and myoepithelial cells **(Fig. 2a)**. In some areas the epithelial cells were cuboidal, with eosinophilic cytoplasm and normal nuclei while the abluminal myoepithelial cells were spindle with more hyperchromatic nuclei. There was no evident pleomorphism, cellular atypia or mitoses **(Fig. 2b).** These microscopical aspects were suggestive of PA.

Nevertheless in other regions, the ducts were larger, formed by pleomorphic cells with increased nucleus/cytoplasm ratio, hyperchromatic nuclei and prominent nucleoli **(Fig. 2c, 2d, 2e).** These areas showed a high immunoexpression of Ki-67 (68.7%) **(Fig. 2f)**, whereas the pleomorphic adenoma area showed low immunoexpression of Ki-67 (4.8%)**.** The final diagnosis was of iminimally invasive CXPA, with a malignant component classified as adenocarcinoma not otherwise specified (NOS).

Clinical and imagenological investigations to detect eventual metastasis were negative. Surgical margins were exiguous, but the patient denied complementary surgical treatment. After a follow up of 30 months, there are no evidences of recurrence or metastases.

Tumor DNA was extracted from paraffin embedded tissue samples using Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) in accord with the manufacturer's recommendations. The carcinomatous and benign area was extracted using a punch with 3mm in diameter, but a good concentration and quality of DNA were obtained only of malignant area. To improve the quality of the isolated DNA, we have applied an elaborate extraction protocol especially for paraffin tissues, which includes thorough deparaffination with xylene, methanol washings to remove all traces of the xylene, and 24-hour incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet was dried and digested for 3 days in lysis buffer with high doses of
proteinase K (final concentration, 2 lg/lL, freshly added twice a day).

The MLPA was performed as described in detail previously using the probe mixture ''SALSA MS-MLPA ME001-C1 tumor suppressor probemix" (MRC-Holland, Amsterdam, the Netherlands; www.mlpa.com). The 41 genes, chromosomal regions and probe sequences are listed in **Table 1**. This mix was chosen to enable the analysis of genes that according to literature may be involved in carcinogenesis[16,17]. Each probe is composed of two parts that hybridize to adjacent target sequences in the DNA. After a ligation step and a PCR amplification, each probe gives rise to a product with a unique size between 136 and 481 nt. The products from PCR reaction were subsequently analyzed on ABI Prism 3100 sequencer and by Peak Scanner v1.0 software (Applied Biosystems, Warrington, UK).

Four different normal salivary glands were used as reference calculating median values and the standard deviations for every probe. For every gene, the relative copy number was calculated by dividing the average relative peak area of the tumor by the median relative peak area of the normal reference samples. It was observed that a MLPA experiment analyzing a new normal DNA resulted in relative copy numbers varying between 0.85 and 1.15, including the standard deviation, for each probe. Therefore, it was

decided that relative copy number values, including the standard deviation, lower than 0.7 were interpreted as losses, higher than 1.3 as gains and 2.5 or higher as amplifications. This interpretation of MLPA data is in accord with Moerland et al. (2006)[18] who found a 98% concordance with data obtained by FISH spot counting, both performed on paraffin tumor material. *P*-values <0.05 were considered significant.

The alterations detected in the current case were loss of *BCL2* (18q21.3), *CASP8* (2q33.2), *CD44* (11p12), *CDH1* (16q22.1), *DAPK1* (9q22), *ESR1* (6q25.1), *RASSF1* (3p21.3), and *TP73* (1p36). The general findings are showed in the **Table 1** and **Graph 1**. The genes *AI651963*, *KLK3*, *FHIT*, *BRCA2*, *IGSF4*, *CDH13*, *TNFRSF7*, *GSTP1*, *MLH1*, *CTNNB1*, *CASR* were not amplified.

DISCUSSION

Typically, CXPA of the minor salivary gland is poorly circumscribed and infiltrative, measuring about 2cm, as current case. The gross appearance depends on the proportions of benign and malignant elements. Histological diagnosis of CXPA can be a challenge, especially if the lesional sample is small as frequently occurs in oral tumors. The CXPA cases should be classified in according to invasiveness and histopathological subtypes. Our case although uncapsulated, it was well circumscribed and delimited, and considered as minimally invasive.

A wide surgical excision with adequate margins is the basic treatment for malignant minor salivary gland tumors. Postoperative radiotherapy is reserved for patients with large primary lesions, perineural or bone invasion, cervical lymph node metastasis, and positive margins. Chemotherapy is used when there is disseminated disease[3]. Our patient had a CXPA involving only the upper lip that was clinically diagnosed and treated as a benign lesion. With the histological diagnosis of malignancy, it was strongly suggested to the patient a complementary surgery to remove eventual residual tumor and increase the margins. Nevertheless the patient did not accept the proposed treatment. After a follow up of 30 months there were no evidences of recurrence or metastasis.

The evolution in transformation from a PA to CXPA requires changes in several steps in cancer hallmarks pathways. The lack of cellular cycle control by inactivation of

tumor suppressor genes as *P16, TP53, RASSF1, RB, hTERT, WT1, K-ras,* shows important association with as CXPA carcinogenesis[13-15]. The current case shows alterations of eight tumor suppressor genes along eight different chromosomes: *BCL2* (18q21.3), *CASP8* (2q33.2), *CD44* (11p12), *CDH1* (16q22.1), *DAPK1* (9q22), *ESR1* (6q25.1), *RASSF1* (3p21.3), and *TP73* (1p36).

The *BCL2* gene encodes a family of apoptosis regulator proteins and it is expressed in many types of malignant tumors protecting cells. A weak positivity for bcl-2 protein in the carcinoma area of CXPA, and strong in the PA areas was found[19-20], showing a role of bcl-2 protein in the development of adenomas, but lost expression in CXPAs, as current case.

The *CASP8* gene encodes a member of the cysteine-aspartic acid protease (caspase). Sequential activation of caspases plays a central role in apoptosis. *CASP8* gene is essential for defense mechanism against hyper-proliferation and tumorigenesis. Polymorphisms in this gene have been reported to influence cancer risk[21]. Essential role of caspase8 protein was described in head and neck carcinoma cells[22], but there is no study of *CASP8* gene and caspase8 protein in CXPA carcinogenesis.

The *CD44* gene encodes a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. The expression of CD44 protein was investigated by Yang et al. (2010)[23] and the authors concluded that the decrease of CD44 expression promoted the recurrence and carcinogenesis of CXPA. Franchi et al. (2001)[24] found that the loss of CD44v3 and CD44v6 protein were associated with the onset of CXPA, and could promote stromal invasion, eventually contributing to the development of distant metastases. Our findings support loss of *CD44* as a malignancy-associated alteration.

The *CDH1* gene encodes cadherin superfamily protein. Mutations in this gene are correlated with gastric, breast, colorectal, oral, thyroid, and ovarian cancers. Loss of function contributes to progression in cancer by increasing proliferation, invasion, and/or metastasis[25]. There are no studies of *CDH1* gene in carcinogenesis of CXPA.

The *DAPK1* (death associated protein kinase 1) gene encodes a protein, that is a positive mediator of gamma-interferon induced programmed cell death, autophagy, and tumor invasion[26-27]. The methylation of this gene as *ESR1* gene is associated with

progression in head and neck squamous cell carcinoma The *ESR1* gene encodes an estrogen receptor that is important for hormone binding, DNA binding, and activation of transcription[16].

The *RASSF1* gene encodes a Ras association domain-containing protein 1. The loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers. The protein inhibits the accumulation of cyclin D1, and thus induces cell cycle arrest. Schache et al. (2010)[15] described that *RASSF1* gene was the single gene promoter for which methylation is shown to be a statistically significant predictor of CXPA.

The *TP73* gene encodes a member of the p53 family of transcription factors involved in cellular responses to stress and development. The over-expression of *TP73* promotes a growth arrest and/or apoptosis. It may be deleted in various tumor entities and human cancers[28], and there is nothing about participation in CXPA.

In conclusion, this case illustrates that unexpected malignancy may occur in unusual location. In addition, the alterations in tumor suppressor genes this case by MLPA, can contribute for understanding of CXPA carcinogenesis.

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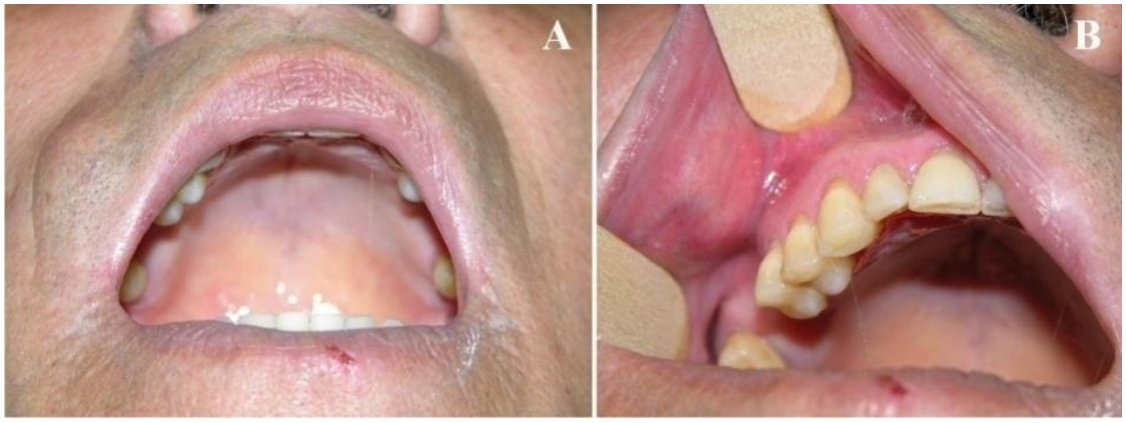
FIGURES



Figs. 1- A and B-Clinical aspects of CXPA involving upper lip (right side).

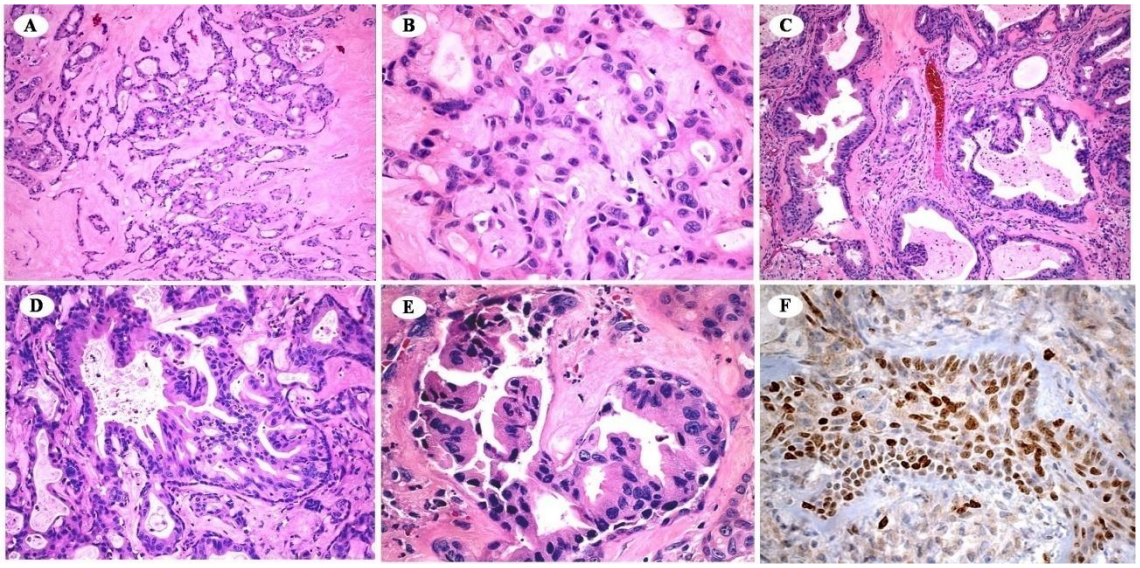


Fig. 2- Carcinoma ex-pleomorphic adenoma of the upper lip. A- Area of PA showing intense hyalinization, with clusters and nests of epithelial cells forming typical ductal structures of PA (H&E, x100). **B-** Epithelial cells forming ducts, and presenting mild nuclear pleomorphism (H&E, x200). **C-E–** Areas of malignant transformation showing large and irregular ducts formed by hyperchromatic pleomorfic cells (H&E, x400). **F-** High nuclear immunoexpression of Ki-67 in the malignant cells forming a solid island.

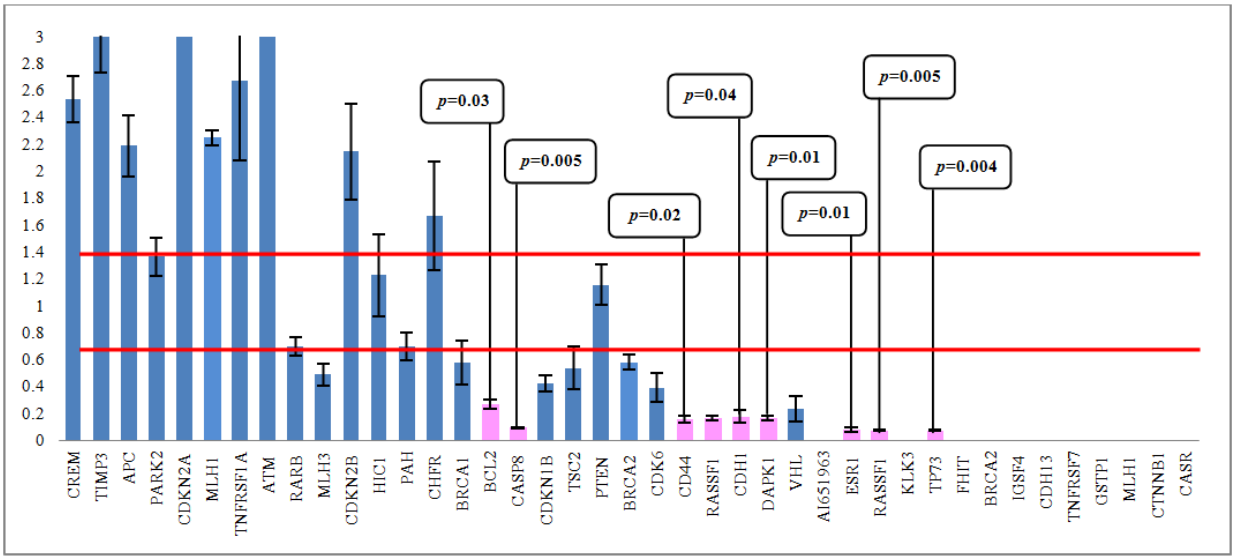
Table 1 – SALSA MS-MLPA ME001- "C1 tumor suppressor probemix" (MRC-Holland, Amsterdam, the Netherlands; www.mlpa.com) applied in current case.

Genes	Chromosomal region	Probe sequence	Gain/loss	*p*=0.05
CREM	10p12.1	GCTCCTCCACCAGGTGCTACAAT	2,532865	0,172682
TIMP3	22q12.3	TCCAGCGCCGAGGCAGCCTCGC	3,622244	0,892368
APC	5q22	CTCAGCTGTGTAATCCGCTGGATGCGGACC	2,188288	0,226996
PARK2	6q26	CGTTCACGACCCTCAACTTGGCTACT	1,36268	0,143438
CDKN2A	9p21	CAGAGGGGAAGAGGAAAGAGGAAGAAGCGCTCAGAT	4,946586	0,590262
MLH1	3p22.1	CGTTGAGCATCTAGACGTTTCCTTGGCTCT	2,245843	0,057424
TNFRSF1A	12p13	TGCCACACTGCCCTGAGCCCAA	2,672401	0,596762
ATM	11q23	GGAGGGAGGAGGCGAGAGGAGTCGGGA	4,118871	0,2386
RARB	3p24	CCGCCGGCTTGTGCGCTCGCT	0,694583	0,067995
MLH3	14q24.3	GCGACCTTGTTCTTCCTTTCCTTCCGA	0,488215	0,082213
CDKN2B	9p21	CTGCGACAGCTCCTGGAAGCCGG	2,142268	0,353573
HIC1	17p13.3	CCGCTCCAGATAAGAGTGTGCGGA	1,224127	0,306472
PAH	12q23	CAGTGCCCTGGTTCCCAAGAA	0,69783	0,104174
CHFR	12q24.33	CGCGAGAGTAGGCGCGTGGAGG	1,666117	0,405742
BRCA1	17q21	GTTCTCAGATAACTGGGCCCCTGC	0,575619	0,16082
BCL2	18q21.3	CTTCTCCTGGCTGTCTCTGAAGACTC	**0,267281***	**0,03515***
CASP8	2q33.2	CTTTCCAATAAAGCATGTCCAGCGCTC	**0,093571***	**0,005402***
CDKN1B	12p13.2	CAGCCCCTGCGCGCTCCTAGA	0,42242	0,061751
TSC2	16p13.3	GAGCCAGAGAGAGGCTCTGAGAAGAAG	0,533916	0,158397
PTEN	10q23.3	CACCGGAGCGGGCGCAGGAGA	1,151949	0,150278
BRCA2	13q12.3	CGGGAGAAGCGTGAGGGGACAGATTTGTG	0,578322	0,058463
CDK6	7q21.3	GCGTGATTGGACTCCCAGGAGAAGAAGACT	0,389723	0,105289
CD44	11p12	CTCCTTTCGCCCGCGCCCTCC	**0,154174***	**0,027022***
RASSF1	3p21.3	CAGTCCCTGCACCCAGGTTTCCA	**0,164012***	**0,019109***
CDH1	16q22.1	CTATGAAGGAAGCGGTTCCGAAGCTGCTA	**0,175978***	**0,046324***
DAPK1	9q22	CGCGAGGATCTGGAGCGAACTGCT	**0,166015***	**0,018447***
VHL	3p25.3	GCGAAGACTACGGAGGTCGACTCGGG	0,230296	0,093986
AI651963	10p14	CAATTGCCATTTTTTCCTGACATTCACTGT	0**	0**
ESR1	6q25.1	CGCCCGCCGTGTACAACTACCCCG	**0,077711***	**0,013292***
RASSF1	3p21.3	GTCCACAGGGCGGGCCCCGAC	**0,072722***	**0,005532***
KLK3	19q13	TGTGTCACCATGTGGGTCCCG	0**	0**
TP73	1p36	CGCCCGCGAAGGGGACGCAGC	**0,071952***	**0,004428***
FHIT	3p14.2	CGCGGGTCTGGGTTTCCACGC	0**	0**
BRCA2	13q12.3	GGCCATGGAATCTGCTGAACAAAA	0**	0**
IGSF4	11q23	CCTGGAGCCCGAGTCCTTGCACGCCA	0**	0**
CDH13	16q24.2	GTTCTGTGCGTTCTCCTGTCCCAG	0**	0**
TNFRSF7	12p13	GAAAGTCCTGTGGAGCCTGCA	0**	0**
GSTP1	11q13	CGAAGAGCGGCCGGCGCCGTG	0**	0**
MLH1	3p22.1	CTGCTGAGGTGATCTGGCGCAGA	0**	0**
CTNNB1	3p22	GGCTGTTAGTCACTGGCAGCAACA	0**	0**
CASR	3q21	CCAGTGCCTGTAACAAGTGCCCAGATGACT	0**	0**

* copy number loss; **no amplification.

GRAPH

Graph 1 – Loss of copies number of tumor suppressor genes of current CXPA.



CONCLUSÕES

-Em nossa série de 38 casos de CXPA, não encontramos diferença entre os gêneros. A média de idade foi de 57 anos, com prevalência maior dos tumores na glândula parótida. O subtipo histopatológico mais frequente foi carcinoma de ducto salivar, seguido do adenocarcinoma NOS, sendo que a maioria dos casos eram francamente invasivos. Um estágio mais avançado da doença foi observado nos casos passíveis de avaliação, e apesar deste fato, baixa taxa de recorrência e mortalidade foi encontrada. As características gerais apresentadas mostram similaridade dos casos às séries apresentadas na literatura.

-Observamos que o índice de Ki-67 é uma ferramenta útil para distinguir AP e CXAP, mesmo na fase precoce. Além disso, o índice de Ki-67 varia entre os diferentes subtipos histopatológicos e invasividade, mas não é estatisticamente significativo, ressaltando a importância dos aspectos morfológicos na diferenciação e classificação.

-Os resultados indicam que vários genes supressores de tumor, ainda não descritos, são alterados durante a tumorigênese do AP e carcinogênese do CXAP. Apesar de morfologicamente semelhantes, o AP e áreas de AP em CXAP, mostram diferenças genotípicas, sendo encontradas alterações significativamente maiores nas áreas de AP em CXAP. Com a transformação maligna e invasividade do CXPA, há um aumento cumulativo de alterações genômicas, que parece ser específico para cada fase, devendo ser melhor exploradas.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Committee of Medical Journal Editor – Grupo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

ANEXO I – CERTIFICADO CEP HOSPITAL AC CAMARGO

Hospital A.C.Camargo
Centro de Tratamento, Ensino e Pesquisa em Câncer

Comitê de Ética em Pesquisa - CEP

São Paulo, 14 de Fevereiro de 2011.

Ao
Dr. Luiz Paulo Kowalski

Ref.: Projeto de Pesquisa nº. 1491/10
"Perfil molecular de carcinoma ex adenoma pleomorfo baseado na análise de oncogenes (fatores de crescimento) por multiplex ligation-dependent probe amplification".

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente - Hospital do Câncer - A.C. Camargo/SP, em sua última reunião de 08/02/2011, após analisarem as respostas aos questionamentos realizados em reunião de 07/12/2010, **aprovaram** a realização do estudo em referência e tomaram conhecimento dos seguintes documentos:

- Folha de Rosto para Pesquisa Envolvendo Seres Humanos;
- Termo de Compromisso do Pesquisador com as Resoluções do Conselho Nacional de Saúde;
- Termo de Dispensa do Consentimento Livre e Esclarecido;
- Declaração sobre os Dados Coletados, Publicação dos Dados e Propriedade das Informações Geradas;
- Declaração sobre Uso e Destino do Material Biológico, Publicação dos Dados e Propriedade das Informações Geradas;
- Orçamento Financeiro Detalhado;
- Declaração de Ciência e Comprometimento do Departamento de Diagnóstico Oral/Patologia da Faculdade de Odontologia de Piracicaba/UNICAMP
- Declaração de Ciência e Comprometimento do Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia do Hospital AC Camargo;
- Declaração de Ciência e Comprometimento do Departamento de Anatomia Patológica do Hospital AC Camargo.

Informações a respeito do andamento do referido projeto deverão ser encaminhadas à assistente do CEP dentro de 12 meses.

Atenciosamente,

Dr. Jefferson Luiz Gross
1º Vice-Coordenador do Comitê de Ética em Pesquisa

1/1

PARECER CONSUBSTANCIADO

Projeto 1491/10

Título: "Perfil molecular de carcinoma ex adenoma pleomorfo baseado na análise de oncogenes (fatores de crescimento) por multiplex ligation-dependent probe amplification"

Pesquisador Responsável: Dr. Luiz Paulo Kowalski

Co-Pesquisadores: Prof. Doutor Oslei Paes de Almeida;
Profª. Dra Albina Messias Almeida Milani Altemani.

Instituição vinculada: Faculdade de Odontologia de Piracicaba

Aluna: Fernanda Viviane Mariano (Doutorado)

Patrocinador: CEPID

Comentários gerais:

Carcinoma ex-adenoma pleomorfo é tumor maligno que pode afetar glândulas salivares maiores e menores. É de ocorrência rara, geralmente manifestada a partir da sexta década de vida, sendo neoplasia de comportamento agressivo e patogênese ainda pouco estudada e determinada. Acredita-se que possa ter origem, em algumas situações após acúmulo de alterações que levem a instabilidade genética em adenomas pleomorfos por períodos prolongados. Discute-se a patogênese desta neoplasia, sendo que alguns defendem que a mesma tenha comportamento maligno desde o início, enquanto há também a discussão de progressão carcinomatosa a partir de componente benigno caracterizado pelo adenoma pleomorfico.

Pendências apontadas em parecer anterior:

1) o título fala de análise da super-expressão, enquanto que o objetivo fala de análise de número de cópias. Acreditamos que o título está incorreto, e deve ser modificado, já que alteração de número não se relaciona obrigatoriamente com aumento ou diminuição da expressão.

2) Não está claro se estudos epigenéticos serão realizados nas amostras, apesar de isto ser desejável, e possível com MLPA.

3) Ainda é preocupante que apenas 10 casos de CaExAP e 10 de AP sejam disponíveis. A análise de alguns genes neste N amostral seria suficiente para a conclusão de um doutoramento em nível de excelência, como deve ser esperado?

4) A técnica a ser usada (MLPA) foi pobremente descrita no projeto, e deveria ser mais cuidadosamente apresentada, deixando claro as suas vantagens e desvantagens em relação a outras técnicas que poderiam ser utilizadas com o mesmo fim.

Análise da Comissão de Pesquisa: Aprovado

Comentários finais:

Todos os questionamentos levantados anteriormente pela Comissão de Pesquisa foram esclarecidos na versão atual do projeto. Portanto, decidimos pela aprovação final do projeto.

Parecer Final:

Projeto Aprovado

ANEXO II – CERTIFICADO CEP FOP

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Perfil molecular de carcinoma ex adenoma pleomorfo baseado na análise da superexpressão de oncogenes (fatores de crescimento) por multiplex ligation dependent probe amplification"**, protocolo nº 002/2011, dos pesquisadores Luiz Paulo Kowalski, Albina Messias de Almeida Milani Altemani, Fernanda Viviane Mariano e Oslei Paes de Almeida, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 11/01/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Molecular profile of carcinoma ex pleomorphic adenoma based on analysis of the overexpression oncogenes (growth factors) for multiplex ligation dependent probe amplification"**, register number 002/2011, of Luiz Paulo Kowalski, Albina Messias de Almeida Milani Altemani, Fernanda Viviane Mariano and Oslei Paes de Almeida, 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 01/11/2011.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

ANEXO III

MATERIAIS E MÉTODOS

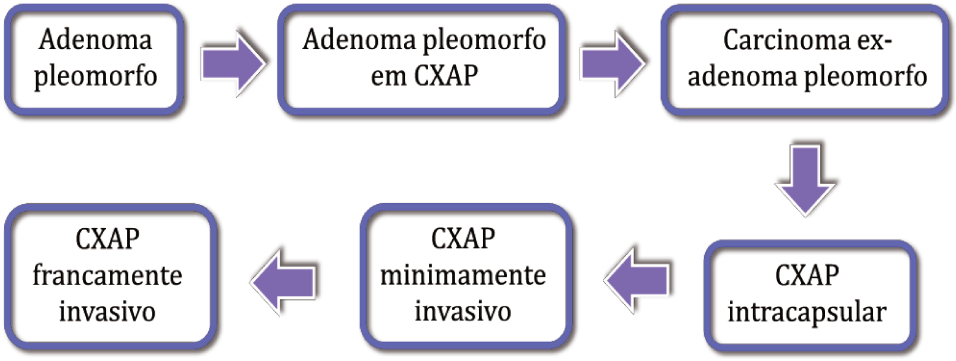
A pesquisa foi realizada no Departamento de Anatomia Patológica da Faculdade de Ciências Médicas-UNICAMP e no Departamento de Diagnóstico Oral/Patologia da Faculdade de Odontologia de Piracicaba-UNICAMP.

Coleta de material

Os pacientes incluídos são portadores de AP ou CXAP, sendo provenientes do Departamento de Diagnóstico Oral – Patologia da FOP/UNICAMP, do Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia do Hospital AC Camargo e do Departamento de Anatomia Patológica da FCM/UNICAMP.

Foram incluídos os casos que possuíam tecido emblocado em parafina, que em estado bem preservado e em quantidade adequada. Eles foram revisados histologicamente e quando possível, toda informação clínica foi coletada para correlação clínica, histopatológica e molecular.

Os CXAPs foram estadiados clinicamente segundo a Classificação dos Tumores Malignos (TNM) proposta pela União Internacional Contra o Câncer (UICC) (Hermaneck, 2002) e os tumores foram analisados histopatologicamente e classificados de acordo com a Organização Mundial da Sáude (World Health Organization Classification of Tumors) (Eveson *et al.,* 2005) quanto aos subtipos histopatológicos e invasividade (intracapsular, minimamente invasivo e francamente invasivos). As análises das lâminas em H&E foram realizadas por três pesquisadores (Fernanda Viviane Mariano, Oslei Paes de Almeida e Albina Messias de Almeida Milani Altemani). Foi feita seleção da área mais representativa de cada tumor, AP e CXAP. Em vista da carcinogênese do AP para CXAP e progressão tumoral do CXAP, pudemos avaliar em nosso estudo, as diferentes fases, como mostrado no esquema a baixo:



Extração do DNA

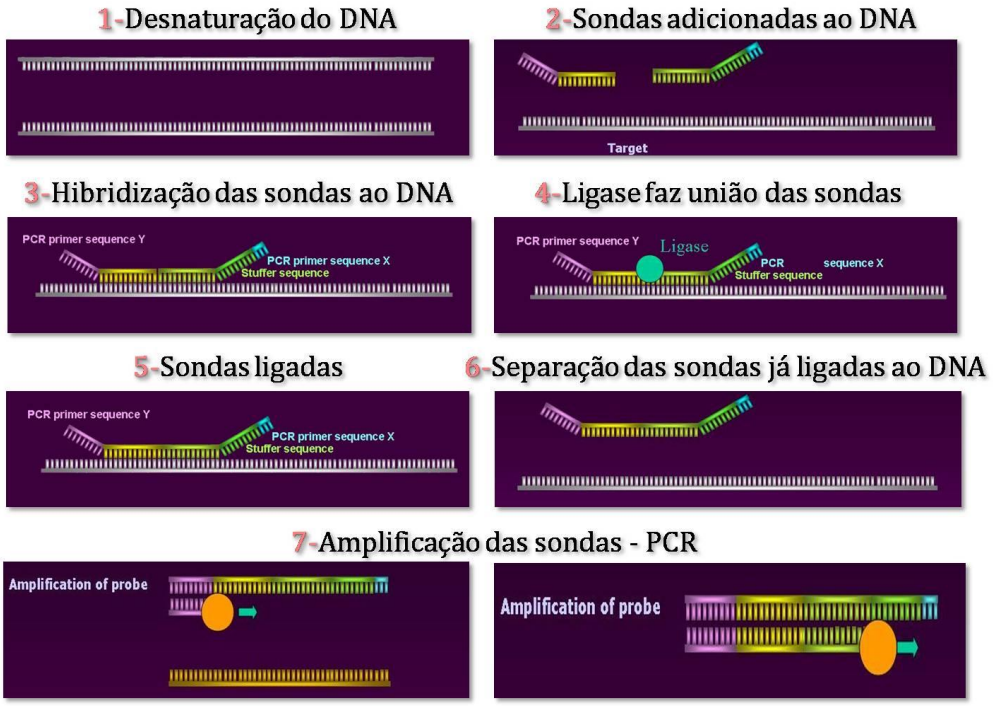
Para a extração do DNA do tecido emblocado em parafina, a área de interesse selecionada através do H&E foi puncionada com auxílio de um punch dermatológico com 5 mm de diâmetro. O DNA foi extraído pela desparafinização em Xilol, lavagem em Metanol e tampão PBS, seguindo com incubação "overnight" em sulfocianato de sódio (NaSCN 1M). A digestão foi feita com proteinase K e a purificação com o uso do QiAmp Mini-kit columns (Qiagen GmbH, Hilden, Germany). Controles normais de DNA foram obtidos de tecidos normais parafinados.

Multiplex Ligation-Probe Dependent Amplification (MLPA)

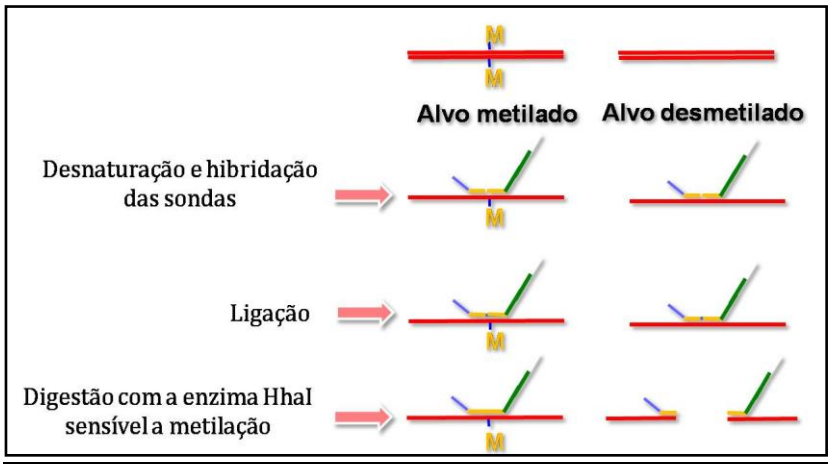
A escolha das sondas de MLPA para este trabalho foi baseada em pesquisa da literatura. A partir destes dados foi escolhido o melhor conjunto de sondas fornecido pela empresa (MRC-Holland, Amsterdam, Netherlands; www.mlpa.com), isto é, o kit que possuísse maior número de genes envolvidos nas alterações moleculares já descritas no CXAP e que fornecesse a possibilidade de estudá-los tendo em vista alterações do número de cópias e metilação. O kit "SALSA MS-MLPA ME001-C1 tumor suppressor probemix" atendia a estas exigências, e portanto foi o escolhido.

Na técnica de MLPA, cada sonda é composta por duas partes que hibridizam sequências alvos adjacentes do DNA. A ligação entre as duas partes da sonda é realizada por uma ligase termoestável. Para a desnaturação do DNA, somente 100ng de DNA são necessários a 98ºC por 5 minutos. A hibridização do DNA com a sonda de MLPA é feita a 60ºC por 16 horas. Cada sonda tem a mesma seqüência final e dá origem a um produto de

tamanho único, que pode variar entre 130 e 480 pares de base (bp), devido a diferença de tamanho da seqüência interna. A amplificação por PCR é realizada a 60ºC por 1minuto, 33 ciclos de a 95ºC por 30segundos, 60ºC por 30 segundos, 72ºC por 1 minuto, seguido por 20 minutos a 72ºC. Os produtos serão mantidos a 4ºC. Toda a técnica é esquematizada a seguir:



As sondas de MLPA para quantificação da metilação são similares às sondas normais de MLPA para detecção do número de cópias, exceto pelo fato de que a sequência detectada pela sonda de metilação é reconhecida pela enzima de restrição sensível à metilação (Hhal). A reação foi realizada em dois tubos sendo que um deles será processado somente com a reação de hibridização acima descrita, que fornecerá informações sobre alterações no número de cópias. No outro tubo a reação de hibridização ocorrerá ao mesmo tempo da digestão das sondas não metiladas, pois neste haverá incubação com a enzima Hhal. As sondas digeridas não serão amplificadas por PCR e consequentemente não gerarão sinal ao serem analisadas por eletroforese capilar (Sellnor e Taylor, 2004). O processo de metilação está esquematizado a seguir:



Forma de análise dos resultados

As amostras foram analisadas por eletroforese capilar gerando dados lidos posteriormente pelo software Peak Scanner v 1.0 (Applied Biosystems, UK).

Determinação do número de cópias:

Após a eletroforese capilar, a altura relativa de cada pico gerado indicava uma deleção ou duplicação da sequência alvo. Os dados foram normalizados, calculando-se a mediana, o desvio padrão e os valores máximos e mínimos, tanto das amostras controles como dos tumores. A duplicação ou a deleção de um gene somente foi considerada como duplicação se a relação tumor/controle for >1.3, e como deleção se <0.7.

ANEXO IV - FIGURAS

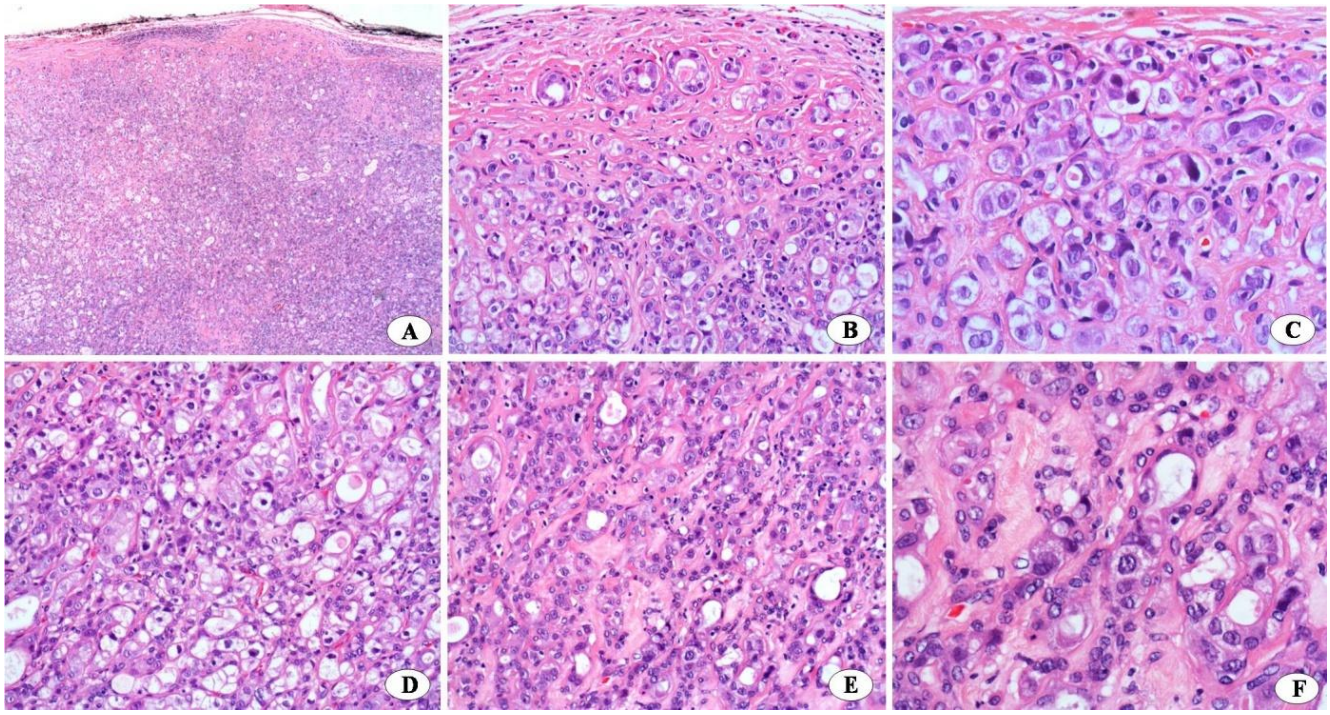


Figura 1- Carcinoma ex-adenoma pleomorfo, do tipo carcinoma de ducto salivar e intracapsular. **A-** Em menor aumento, pode-se notar manutenção das células tumorais delimitadas pela cápsula (H&E x5). **B, C-** Algumas células malignas se extendem em direção à cápsula (H&E x20, x40). **D-** Presença de células epiteliais claras, por vezes vacuolizadas (H&E x20). **E, F-** Os ductos são formados por células epiteliais luminais, apresentando formatos irregulares. As células apresentam extenso pleomorfismo celular com núcleos picnóticos e atípicos (H&E x20, x40).

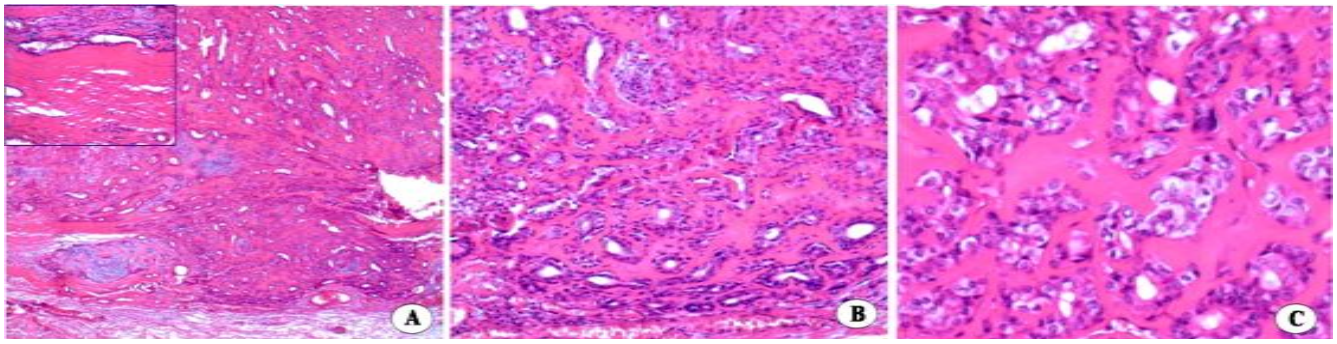


Figura 2- Carcinoma ex-adenoma pleomorfo, tipo epitelial-mioepitelial e minimamente invasivo. **A-** Células tumorais se extendendo para cápsula, porém a uma distância menor que 1,5 mm (H&E x5). **B-** Dupla camada de células, epiteliais ao lúmen e mioepiteliais ao redor formando ductos irregulares e tortuosos (H&E x20). **C-** Pleomorfismo celular e atipia celular (H&E x40).

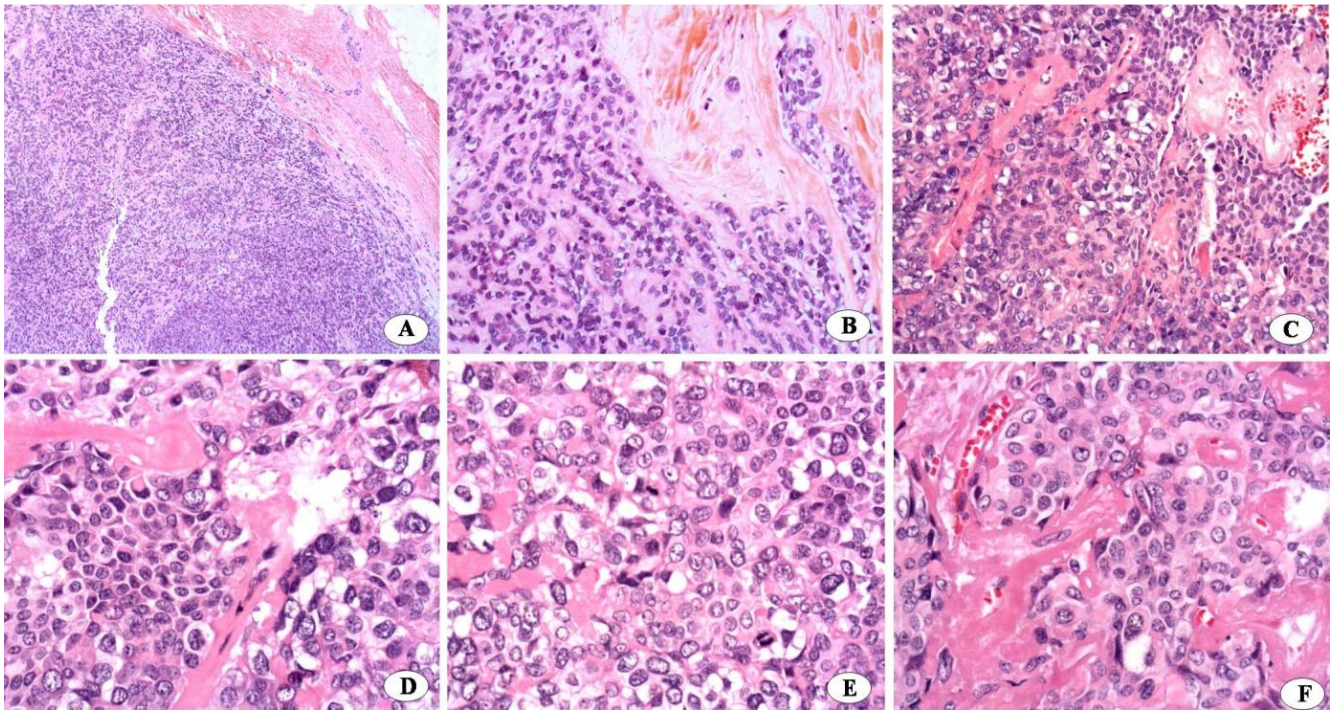


Figura 3- Carcinoma ex-adenoma pleomorfo, tipo mioepitelial e minimamente invasivo. A, B- Lençol de células com ilhas de células extendendo-se em direção à cápsula (H&E x5, x20). **C-** Formação trabecular (H&E x20). **D, E-** Pleormofismo celular, mitoses atípicas, células escuras e outras vacuolizadas (H&E x40). **F-** Intensa matriz hialina e raros ductos (H&E x40).

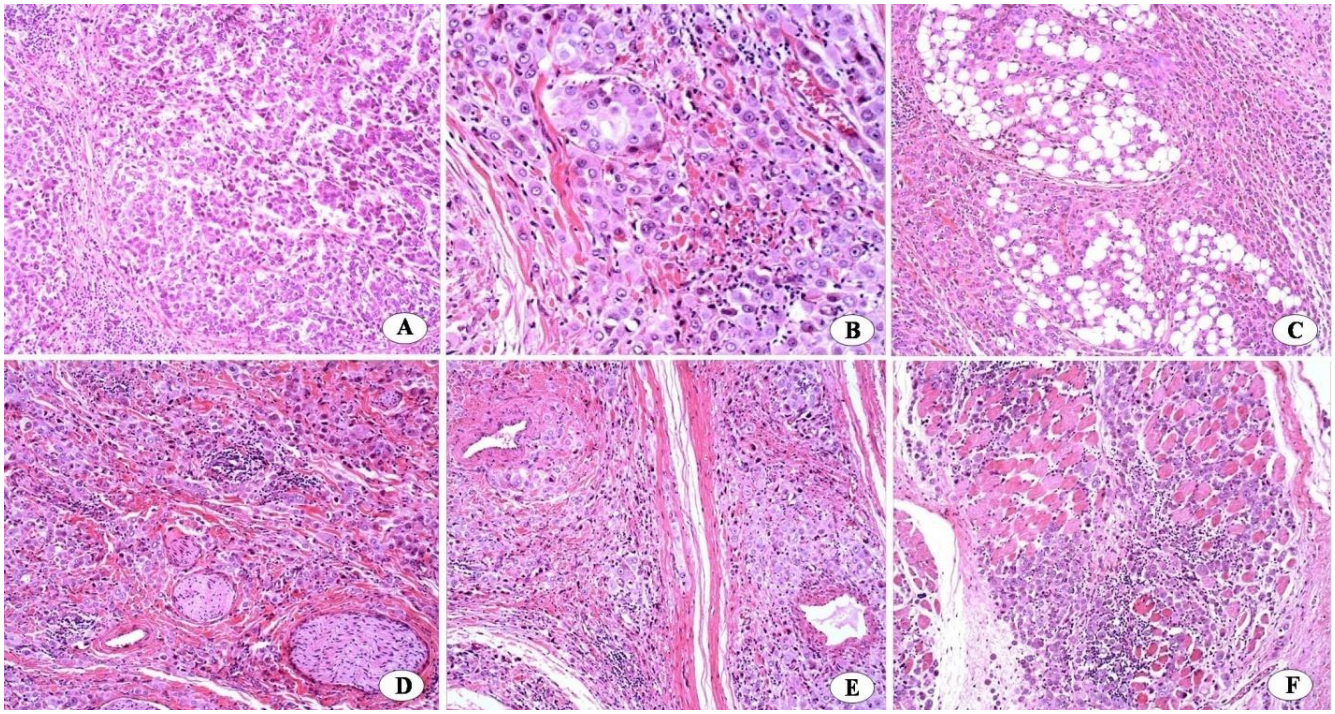


Figura 4- Carcinoma ex-adenoma pleomorfo, tipo adenocarcinoma NOS e francamente invasivo. A, B-Lençol de células epiteliais mostrando atipia celular e formação ductal (H&E x5, x20). **B-F-** Infiltração de estruturas adjacentes: tecido adiposo, neural, vascular e muscular (H&E x5).

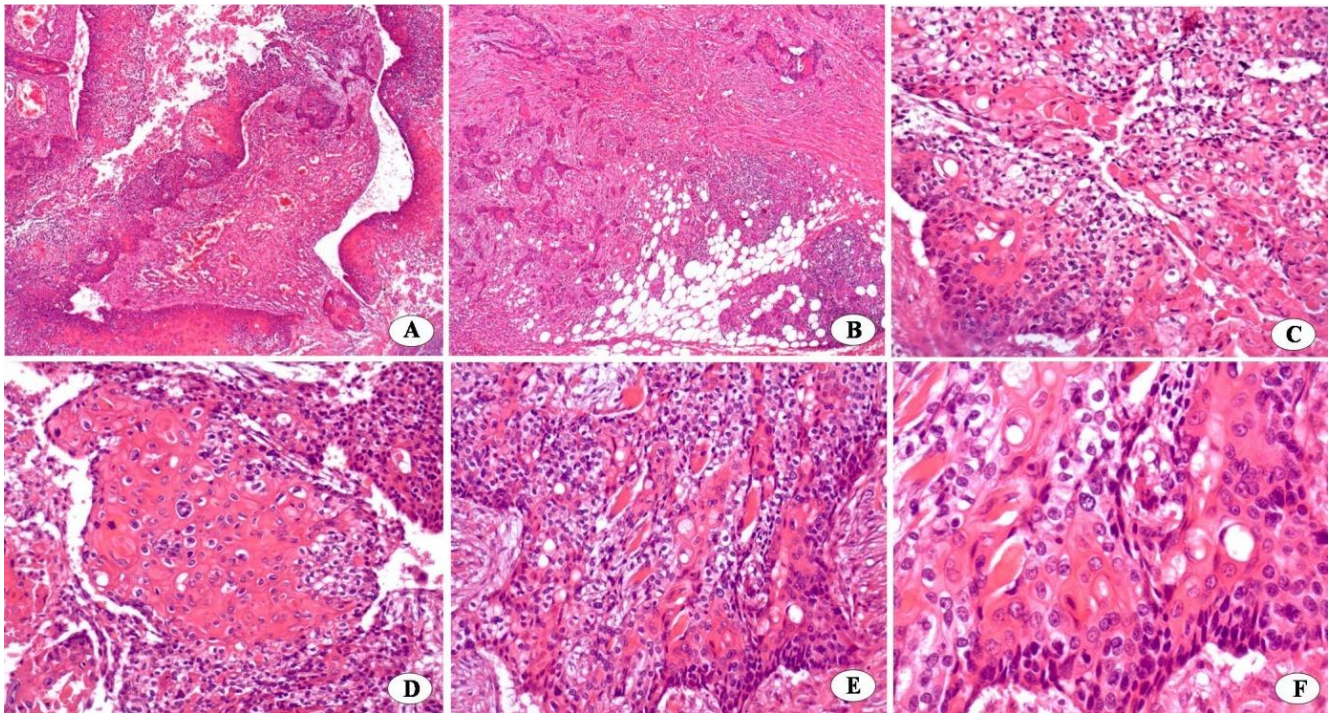


Figura 5 – Carcinoma ex-adenoma pleomorfo, tipo epidermóide e francamente invasivo. A- Proliferação de epitélio escamoso (H&E x5). **B-** Invasão das ilhas escamosas em tecido glandular remanescente (H&E x5). **C-** Células vacuolizadas e formação de pérolas de queratina (H&E x20). **D-** Pleomorfismo celular atipia nuclear (H&E x20). **E, F-** Formação ductal (H&E x20, x40).

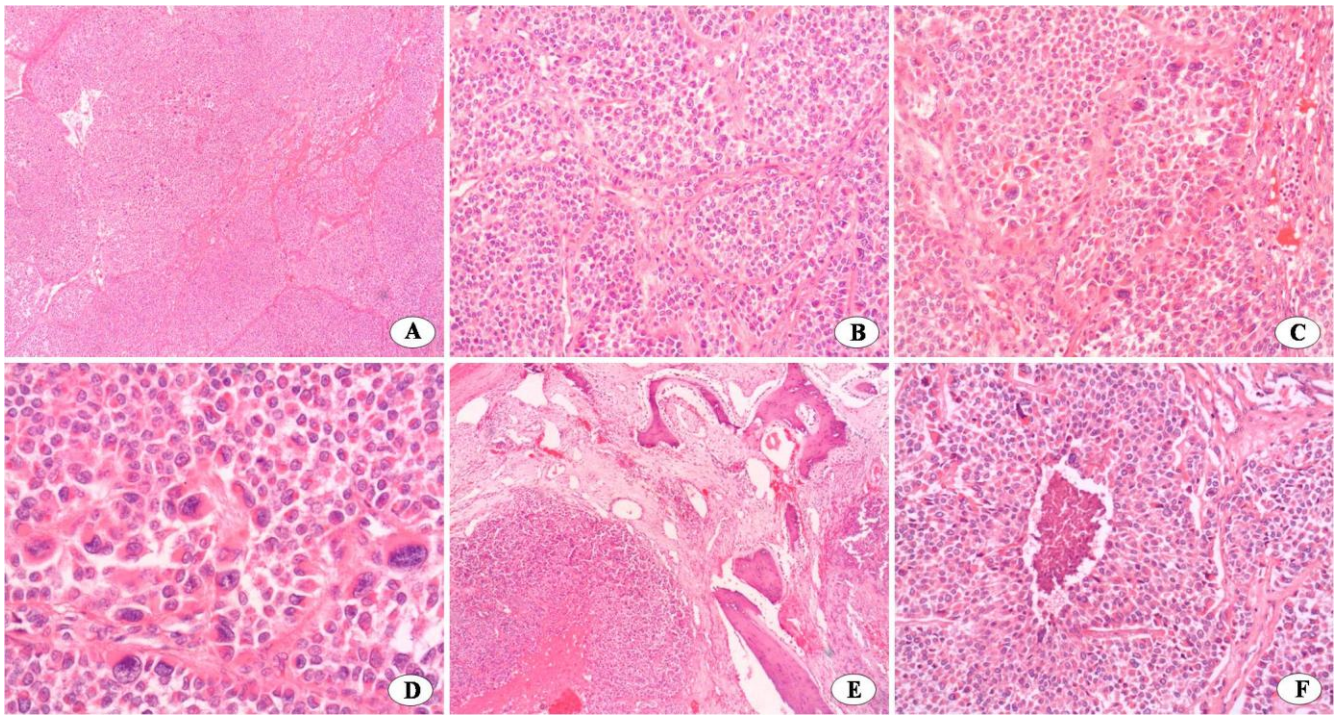


Figura 6- Carcinoma ex-adenoma pleomorfo, tipo mioepitelial e francamente invasivo. A- Arquitetura multilobulada (H&E x5). **B-** Células mioepiteliais dispostas em arranjo sólido em folhas divididas por traves hialinas (H&E x20). **C, D-** Aspecto celular plasmocitóide, mostrando intensa atipia e pleomorfismo celular (H&E x20, x40). **E-** Padrão de crescimento infiltrando osso adjacente (H&E x5). **F-** Necrose central ou comedonecrose (H&E x20).