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ORIGINAL ARTICLE

Carcinoma ex-pleomorphic adenoma derived from recurrent pleomorphic adenoma shows important difference by array CGH compared to recurrent pleomorphic adenoma without malignant transformation☆

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KEYWORDS
Carcinoma ex-pleomorphic adenoma;
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Somatic copy number alterations;
aCGH

Abstract
Introduction: A key step of cancer development is the progressive accumulation of genomic changes resulting in disruption of several biological mechanisms. Carcinoma ex-pleomorphic adenoma (CXPA) is an aggressive neoplasm that arises from a pleomorphic adenoma. CXPA derived from a recurrent PA (RPA) has been rarely reported, and the genomic changes associated with these tumors have not yet been studied.
Objective: We analyzed CXPA from RPAs and RPAs without malignant transformation using array-comparative genomic hybridization (array-CGH) to identify somatic copy number alterations and affected genes.
Methods: DNA samples extracted from FFPE tumors were submitted to array-CGH investigation, and data was analyzed by Nexus Copy Number Discovery Edition v.7.
Results: No somatic copy number alterations were found in RPAs without malignant transformation. As for CXPA from RPA, although genomic profiles were unique for each case, we detected some chromosomal regions that appear to be preferentially affected by copy number

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alterations. The first case of CXPA-RPA (frankly invasive myoepithelial carcinoma) showed copy number alterations affecting 1p36.33p13, 5p and chromosomes 3 and 8. The second case of CXPA-RPA (frankly invasive epithelial-myoepithelial carcinoma) showed several alterations at chromosomes 3, 8, and 16, with two amplifications at 8p12p11.21 and 12q14.3q21.2. The third case of CXPA-RPA (minimally invasive epithelial-myoepithelial carcinoma) exhibited amplifications at 12q13.3q14.1, 12q14.3, and 12q15.
Conclusion: The occurrence of gains at chromosomes 3 and 8 and genomic amplifications at 8p and 12q, mainly those encompassing the HMGA2, MDM2, WIF1, WHSC1L1, LIRG3, CDK4 in CXAP from RPA can be a significant promotional factor in malignant transformation.

PALAVRAS-CHAVE
Carcinoma ex-adenoma pleomórfico;
Adenoma pleomórfico recorrente;
Alterações no número de cópias somáticas;
*a*CGH

Carcinoma ex-adenoma pleomórfico derivado de adenoma pleomórfico recorrente mostra diferença importante por *array* CGH em comparação com adenoma pleomórfico recorrente sem transformação maligna

Resumo
Introdução: Uma etapa fundamental do desenvolvimento do câncer é o acúmulo progressivo de alterações genômicas, resultando na ruptura de vários mecanismos biológicos. Carcinoma ex-adenoma pleomórfico (CXAP) é uma neoplasia agressiva que surge a partir de um adenoma pleomórfico. O CXAP derivado de um AP recorrente (APR) foi raramente relatado e, até o momento, as alterações genômicas associadas a esses tumores não foram estudados.
Objetivo: Avaliar as diferenças entre os CXAPs decorrentes de APRs e os APRs sem transformações malignas usando hibridização genômica comparativa em *microarrays* (array Comparative Genomic Hibridization — aCGH) a fim de identificar alterações no número de cópias somáticas e os genes afetados.
Método: Amostras de DNA extraídas de tumores provenientes de tecido emblocado em parafina foram submetidos à investigação com a técnica aCGH, e os dados foram analisados com o Nexus Copy Number Discovery Edition v.7.
Resultados: Não observamos alterações no numero de cópias somáticas nos APRs sem transformação maligna. Quanto ao CXAP de APR, embora os perfis genômicos sejam exclusivos para cada caso, detectamos algumas regiões cromossômicas que pareciam ser preferencialmente afetadas por alterações no número de cópias. O primeiro caso de CXAP-APR (carcinoma mioepitelial francamente invasivo) apresentou alterações no numero de cópias afetando 1p36.33p13, 5p e cromossomos 3 e 8. O segundo caso de CXAP-APR (carcinoma epitelial-mioepitelial francamente invasivo) apresentou várias alterações nos cromossomos 3, 8 e 16, com duas amplificações em 8p12p11.21 e 12q14.3q21.2. O terceiro caso de CXAP-APR (carcinoma epitelial-mioepitelial minimamente invasivo) apresentou amplificações em 12q13.3q14.1, 12q14.3, e 12q15.
Conclusão: A ocorrência de ganhos de cromossomos 3 e 8, e as amplificações genômicas em 8p e 12q, principalmente aquelas que englobam os HMGA2, MDM2, WIF1, WHSC1L1, RG3, CDK4 no CXAP decorrente de APR podem ser fatores promocionais significativos para a transformação maligna.

Introduction

Pleomorphic adenoma (PA) is the most common tumor of the salivary glands, accounting for about 60–70% of such neoplasms. It is a benign tumor with high risk of recurrence and malignant transformation.[1] The recurrence risk ranges from 0.4% to 45%, depending on the surgical technique[2]: 20–45% after enucleation, 2–5% following parotid lobectomy, and up to 0.4% after radical parotidectomy.[3] Recurrent Pleomorphic adenoma (RPA) presumably derives either from capsule rupture, incomplete resection of microscopic extensions beyond the pseudocapsule, or multifocal origin.[4]

Permanent facial nerve injury risk, multinodular feature, and increased frequency of new recurrence are factors that make the treatment of RPA difficult.[5] Furthermore, the risk of malignant transformation increases with the duration of the disease.[6,7] To date, CXPA arising from RPA has been rarely

reported and these studies have focused on the histopathological and clinical features of the lesions.[8]

The recurrence of tumor can be caused by either increase in the number or complexity of genetic abnormalities or acquisition of promoting mutations to the malignant change. Cancer is driven by somatically acquired mutations, and chromosomal rearrangements are thought to accumulate gradually over time.[9] Whole-genome screening such as array-CGH can be applied to disclose copy number alterations which could identify molecular basis for carcinogenesis.

Herein, the aim of this study was to investigate by array comparative genomic hybridization (aCGH) the genomic profile of copy number alterations associated with three cases of carcinoma ex-pleomorphic adenoma (CXPA) originated from RPA, discover their involved genes, and compare these findings to four cases of RPA without malignant transformation.

Methods

The current study was carried out in accordance with the ethical guidelines of our institution (Process n° CEP/FOP 002/2011). DNA samples were extracted from a 1.5 mm diameter punch of paraffin embedded tumor tissues using Qiagen extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendations. The protocol included deparaffinization with xylene, followed by methanol washings, and 24-hour incubation in 1moL/L sodium thiocyanate. Subsequently, the tissue pellet was dried and digested for 1.5 day in a lysis buffer with high proteinase K level (60 μL). Samples were column-purified before buffer elution.

Tumor and reference DNA (pooled from blood of different healthy donors; Promega, Madison, WI, USA) samples were differently labeled using the Enzo Genomic DNA Labeling kit according to the manufacturer's instructions. Five hundred ηg of labeled tumor and reference DNA were co-hybridized to a 180 K oligonucleotide array (SurePrint G3 Human CGH Microarray Kit 4 × 180 K design 22060, Agilent Technologies, Palo Alto, CA, USA), following manufacturer procedures. This design contains 24,011 exonic probes. Microarray images were obtained by Agilent Microarray Scanner Bundle, and data was extracted using the Feature Extraction software v.9.1 (Agilent Technologies, Santa Clara, CA, USA).

Array-CGH data was analyzed using the software Nexus Copy Number Discovery edition v.7.0. Genomic copy number alterations were called based on the FASST2 segmentation algorithm (significance threshold set on 5×10^{-8}) with threshold $\log_2$ ratios of 0.2 or 0.8 for gains or high-copy gains, respectively, and −0.2 or −1.0 for losses or homozygous losses, respectively.

Results

Clinic-pathological data of the CXPA from RPA

The first patient (case 1) was a 72 year-old man referred to our hospital for evaluation of a nodule in the parotid gland measuring 9.0 cm × 8.0 cm with a reported time of evolution of two years. The patient had undergone resection of a PA five years ago. During clinical examination, palpable lymph nodes and subjacent skin invasion were observed. There was absence of oral lesions. Fine needle aspiration biopsy revealed a PA. Tumor was excised with positive surgical margins. Histological examination showed presence of PA and CXPA regions. The neoplasm was classified as a frankly invasive myoepithelial carcinoma (Fig. 1A and B). The patient was submitted to radiotherapy and no recurrence was observed in 58 months of follow-up.

The second patient (case 2) was a 66 year-old woman referred to our hospital complaining of a tumor in the parotid region for an unknown time. The patient had undergone resection of a PA 11 years ago. Clinical examination rules out the presence of palpable lymph nodes and oral lesions. Fine needle aspiration biopsy confirmed the presence of a PA. Tumor excision was performed but the margins were positive surgically. Histological examination showed presence of PA and CXPA, which were classified as frankly invasive epithelial-myoepithelial carcinoma (Fig. 1C and D). There is no follow-up of this patient.

The third patient (case 3) was a 30 year-old woman referred to our hospital complaining of a tumor in the parotid gland with two years of duration. The patient had undergone resection of a PA 16 years ago. During clinical examination, palpable lymph nodes and oral lesions were not observed. Fine needle aspiration biopsy showed presence of PA. The tumor was excised with negative surgical margins. The histopathological analysis showed PA and CXAP regions. The latter was a minimally invasive epithelial-myoepithelial carcinoma (Fig. 1E and F). There is no follow-up of this patient.

Array-CGH analysis

The cases of RPAs did not show somatic copy number alterations. All somatic chromosomal alterations detected in CXPAs from cases 1, 2 and 3 are detailed in Table 1, as well as affected known cancer genes according to the Cancer Gene Census Sanger (https://www.sanger.ac.uk/research/projects/cancergenome/census.html). Fig. 2 presents the global genomic profile of copy number alterations identified in CXPA cases 1, 2 and 3. The first CXPA from RPA exhibited 1p36.33p13 loss, chromosomes 3 and 8 gains, and two adjacent chromosomal rearrangements affecting 5p15.33p13.1 (loss) and 5p13.1q13.1 (gain), respectively.

The second case of CXPA from RPA showed a more complex genomic pattern with several copy number alterations (gains and losses) affecting chromosomes 3, 8 and 16. Additionally, this sample harbor losses at 5q14.3q33.1, 5q35.3, 10p15.3p13, 14q11.2q32.2, 20q13.12, and gains at 6p22.2, 22q11.1q13. Regions of high copy number gains (amplifications) were found at 8p12p11.21 and 12q14.3q21.2 (Fig. 3A). The amplified genes included among others WHSCILI and FGFR1 at 8p, and HMGA2 and MDM2 at 12q.

The third case exhibited losses at 12q14.1q14.2 and 12q14.3q15, and amplifications at 12q13.3q14.1, 12q.14.3 and 12q15, encompassing CDK4, LRIG3, WIF1, HMGA2 and MDM2 (Fig. 3B).

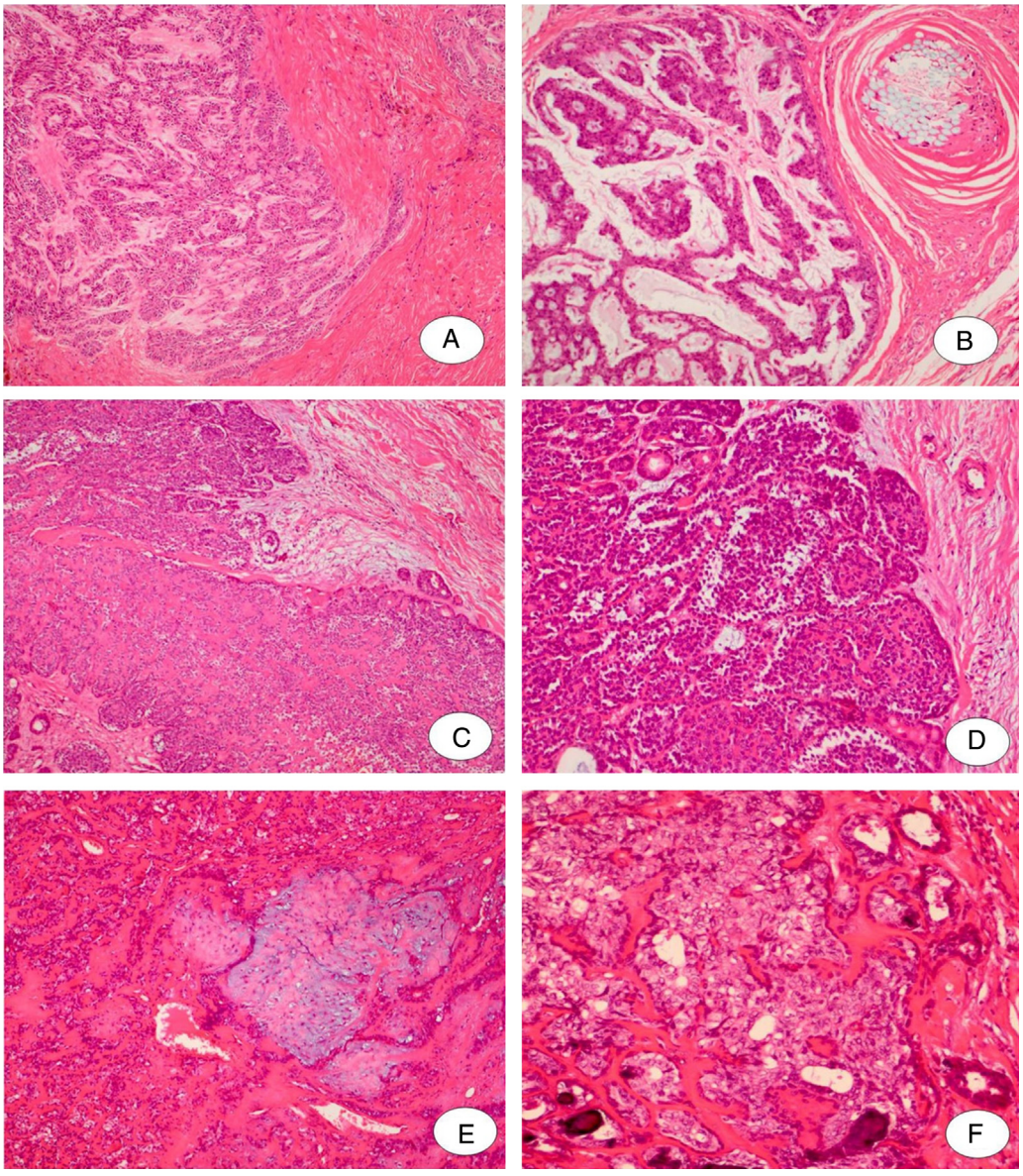

Figure 1 Frankly invasive myoepithelial carcinoma: (A) Island of myoepithelial cells infiltrating the tissue (H&E × 10); (B) cords of pleomorphic myoepithelial cells surrounded by myxoid stroma. Note the reaction against thread suture from previous surgery in the top right side of the image (H&E 20×). Frankly invasive epithelial-myoepithelial carcinoma: (C) Proliferation of epithelial and myoepithelial cells in a nodular growth (H&E 10×); (D) Small lumen bounded by eosinophilic, cuboidal, intercalated duct-like cells. These cells are surrounded by small and non-staining cytoplasm cells. Note the maintenance of basal cells in the periphery of nest cells surrounded by fibrous septa (H&E 20×). Minimally invasive epithelial-myoepithelial carcinoma: (E) Epithelial-myoepithelial proliferation arising in pleomorphic adenoma residual (H&E 10×); (F) eosinophilic, hyalinized basal lamina material surrounds nests of tumor cells and ductal structures comprised of epithelial and myoepithelial cells (H&E 20×).

Discussion

The carcinogenesis occurs in several steps through genomic changes that result in loss of tumor suppressor functions, the activation of oncogenes and/or the generation of fusion genes with oncogenic potential.[10] These alterations generate clonal expansion resulting in phenotype of malignant cancer cells.[11] Although such changes can occur by mutations or genomic rearrangements, abnormal chromosome numbers and structures have also been well reported in neoplastic cells, indicating that chromosome instability is an important aspect of cancer cell biology.[10] Therefore, copy number alterations can be an auxiliary tool in the understanding about carcinogenesis.

Malignant transformation of recurrent pleomorphic adenoma is reported in 1.5–23% of cases, and the risk appears to increase with time and number of recurrences.[12] The occurrence of malignant change from recurrence of the pleomorphic adenoma must involve the acquisition of mutations over a period of time.

The current cases exhibited different patterns of copy number alterations, and it is important to emphasize that

Table 1 Somatic copy number alterations detected by array-CGH in three cases of CXPA from RPA.

Chromosome coordinates (Hg19)	Event type	Size (Mb)	Cytoband	Genes (n)	Known cancer genes (CGCS)
Case 1					
chr1:0-12,034,621-109,356,617	Loss	109	1p36.33-p13.3	1204	TNFRSF14, PRDM16, RPL22, CAMTA1, SDHB, PAX7, MDS2, ARID1A, LCK, SFPQ, THRAP3, MYCL1, MPL, MUTYH, TAL1, CDKN2C, EPS15, JUN, JAK1, FUBP1, BCL10
chr3:0-91,000,000	Gain	91.0	3p26.3-q11.1	646	SRGAP3, FANCD2, VHL, PPARG, RAF1, XPC, MLH1, MYD88, CTNNB1, SETD2, BAP1, PBRM1, FHIT, MITF, FOXP1
chr3:95,011,793-163,987,310	Gain	69.0	3q11.2-q26.1	490	TFG, CBLB, GATA2, RPN1, FOXL2, WWTR1, GMPS, MLF1
chr3:164,108,626-198,022,430	Gain	34.0	3q26.1-q29	261	EVI1, PIK3CA, SOX2, ETV5, EIF4A2, BCL6, LPP, TFRC
chr5:0-40,851,406	Loss	40.8	5p15.33-p13.1	187	IL7R, LIFR
chr5:40,935,588-46,150,843	Gain	27	5p13.1-q13.1	125	IL6ST, PIK3R1
chr8:0-43,647,122	Gain	146	8p23.3-q24.3	926	PCM1, WRN, WHSC1L1, FGFR1, HOOK3, TCEA1, PLAG1, CHCHD7, NCOA2, HEY1, COX6C, EXT1, MYC, NDRG1, RECQL4
Case 2					
chr3:24,527,963-90,336,853	Loss	6.5	3p24.2-p11.1	451	MLH1, MYD88, CTNNB1, SETD2, BAP1, PBRM1, FHIT, MITF, FOXP1
chr3:93,529,103-101,960,258	Gain	0.8	3q11.1-q12.3	54	TFG
chr3:101,960,258-102,594,287	Loss	0.06	3q12.3	1	
chr3:102,610,999-197,939,679	Gain	9.5	3q12.3-q29	628	CBLB, GATA2, RPN1, FOXL2, WWTR1, GMPS, MLF1, EVI1, PIK3CA, SOX2, ETV5, EIF4A2, BCL6, LPP, TFRC
chr5:85,168,149-152,581,242	Loss	6.7	5q14.3-q33.1	438	APC, PDGFRB, CD74
chr5:180,417,510-180,915,260	Loss	0.05	5q35.3	17	
chr6:26,120,677-26,291,646	Gain	0.01	6p22.2	21	
chr8:0-33,163,303	Loss	3.3	8p23.3-p12	255	PCM1, WRN
chr8:35,142,906-39,877,924	Amplification	0.5	8p12-p11.21	36	WHSC1L1, FGFR1
chr8:39,877,924-43,527,965	Gain	0.3	8p11.21-p11.1	28	HOOK3
chr8:47,553,667-146,364,022	Gain	9.8	8q11.1-q24.3	506	TCEA1, PLAG1, CHCHD7, NCOA2, HEY1, COX6C, EXT1, MYC, NDRG1, RECQL4
chr10:0-13,151,933	Loss	1.3	10p15.3-p13	75	GATA3
chr12:66,133,957-76,156,328	Amplification	1.0	12q14.3-q21.2	55	HMGA2, MDM2
chr14:20,595,449-98,566,915	Loss	7.8	14q11.2-q32.2	579	CCNB1IP1, TRA@, NKX2-1, NIN, KTN1, GPHN, TSHR, TRIP11, GOLGA5, DICER1, TCL6, TCL1A
chr16:0-35,147,508	Gain	3.5	16p13.3-p11.1	532	TSC2, CREBBP, CIITA, SOCS1, TNFRSF17, ERCC4, MYH11, PALB2, IL21R, FUS
chr16:46,367,235-90,237,661	Loss	4.3	16q11.2-q24.3	418	CYLD, HERPUD1, CDH11, CBFB, CDH1, MAF, CBFA2T3, FANCA
chr20:45,505,668-46,151,351	Loss	0.6	20q13.12	5	
chr22:17,296,232-51,274,523	Gain	3.3	22q11.1-q13.33	540	CLTCL1, BCR, SMARCB1, MN1, CHEK2, EWSR1, NF2, MYH9, PDGFB, MKL1, EP300
Case 3					
chr12:57,993,000-60,129,343	Amplification	0.2	12q13.3-q14.1	25	CDK4, LRIG3
chr12:60,129,343-64,578,600	Loss	4.4	12q14.1-q14.2	12	
chr12:65,484,807-66,489,652	Amplification	0.1	12q14.3	8	WIF1, HMGA2
chr12:66,489,652-68,720,923	Loss	2.2	12q14.3-q15	17	
chr12:68,720,923-71,009,093	Amplification	0.2	12q15	26	MDM2

Amplification, high copy number gains are presented as genomic amplifications.

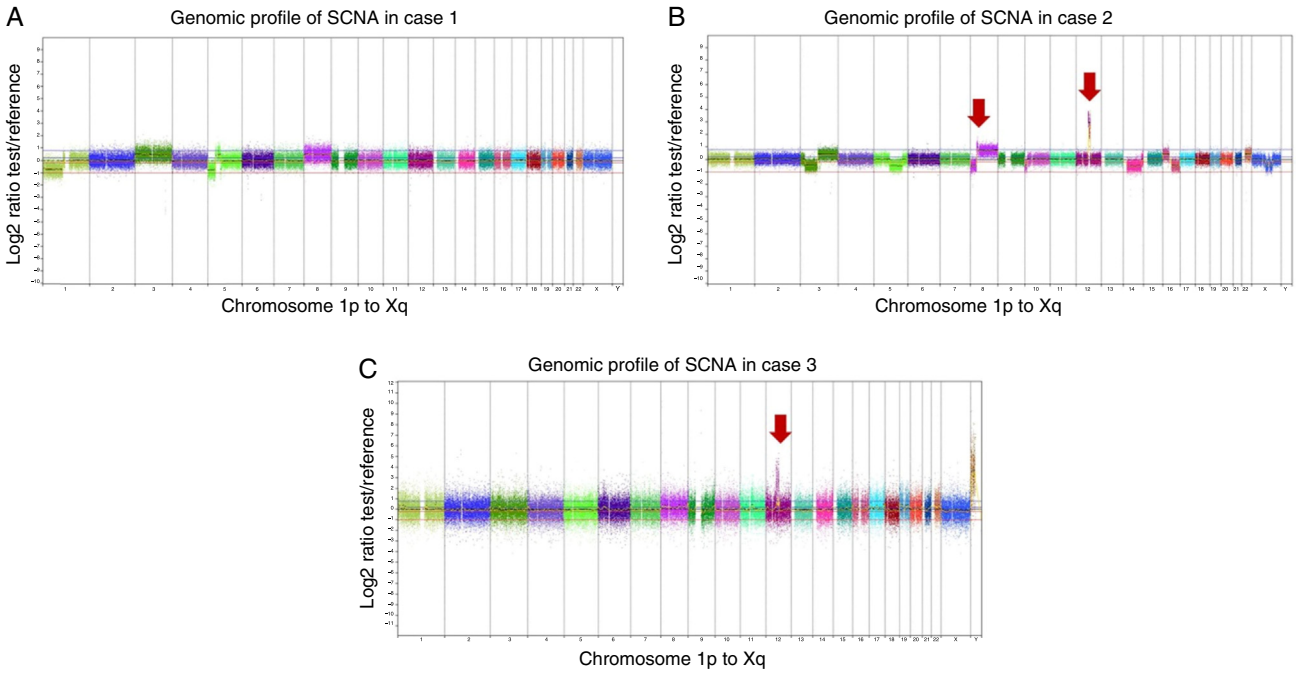

Figure 2 Copy number alterations detected by array-CGH in the CXPAs from RPA cases. Array-CGH genomic profile exhibiting the identified copy number alterations of case 1 (A), case 2 (B) and case 3 (C). The *x*-axis represents probes ordered according to their genomic position from chromosomes 1p to Xq (each chromosome is labeled with a different color). The *y*-axis denotes the $\log_2$ test/reference values (genomic gains and losses are plotted above or below the 0 baseline, respectively; images adapted from the software Nexus Copy Number 7.0, Biodiscovery). The arrows indicate the high copy gains (amplifications).

histopathological and invasiveness classifications were also distinct. However, data analysis could pinpoint some recurrent copy number alterations such as 3q and 8q gains (cases 1 and 2), and importantly, an amplification with a minimum common region at 12q14.3 (cases 2 and 3). Cases 1 and 2 were frankly invasive carcinomas, and 3q and 8q gains can be implicated in the invasiveness degree. Cases 2 and 3 were epithelial-myoepithelial carcinomas, and the 12q14.3 amplification is maybe involved with histopathological subtype, or even recurrence. Case 2 exhibited a more complex pattern of rearrangements consistent with the histopathological subtype and degree of invasiveness.

Losses of 1p21.3-p21.1, 5q23.2-q31.2, 8p, 10q21.3 and 15q11.2 were found by Persson et al.[13] in a study of a group of 10 CXPAs; however, no histopathological classification was performed. We find losses at 1p36.33-p13, 5p15.33-p13.1, 5q14.3-q33.1, 5q35.3, 8p and 10p15.3-p13. Gain of 8q12.1 (PLAG1), here detected in two cases, has been reported by several authors.[13–15]

Amplifications of HMGA2, MDM2, and deletions of 5q23.2q31.2 and 8q22.1q24.1 were described as important in the transition from PA to CXPA.[13] We observed high copy gain of HMGA2, MDM2, CDK4, WHSCIL1, LRIG3 and WIF1. All the amplified genes are cancer related, according to the Cancer Gene Census Sanger (https://www.sanger.ac.uk/research/projects/cancergenome/census.html). HMGA2 and MDM2 amplification were found in two of our cases, reinforcing their role as driver genes associated with recurrence in CXPA.

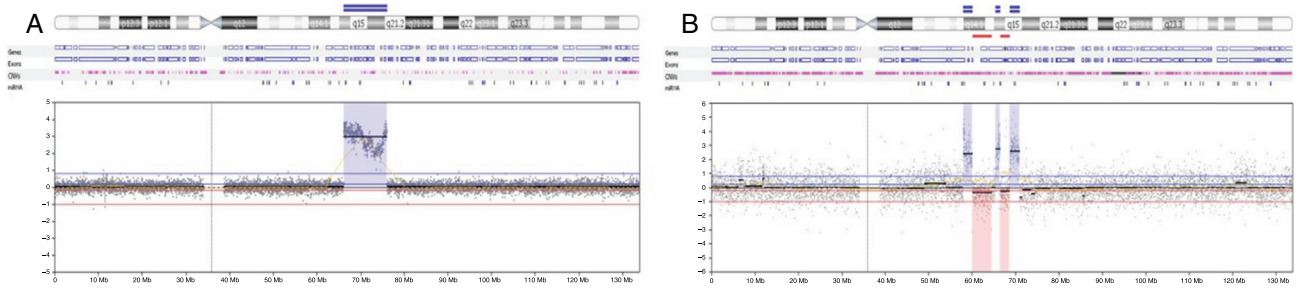

Figure 3 (A) Array-CGH profile of chromosome 12 showing the high copy number gain (amplification) of 1Mb at 12q14.3q21.2 in case 1. (B) Array-CGH profile of chromosome 12 showing a complex pattern consisting of three genomic regions exhibiting high copy number (amplifications) at 12q13.3q14.1 (0.2 Mb), 12q14.3 (0.1 Mb) and 12q15 (0.2 Mb), interpolated with copy number losses of low amplitude.

HMGA2 (human high mobility group A) gene encodes a non-histone chromatin protein that belongs to a family of the HMG proteins, which are overexpressed in malignant neoplasms as lung, pancreatic, oral squamous cell carcinoma and breast cancer.[16–19] HMGA2 proteins have oncogenic activity through several mechanisms, such as induction of E2F1 and AP1 activity, induction of cyclin A expression, inactivation of p53 induced apoptosis, impairment of DNA repair, enhancement of the expression of proteins involved in inflammation, and modulation of the expression of microRNAs and genes involved in epithelial-mesenchymal transition.[20] Additionally, HMGA proteins have a crucial role in cell transformation because when their synthesis is blocked, suppression of the malignant phenotype occurs. This hypothesis is in line with our finding, because we showed the amplification of HMAG2 from a minimally invasive case.

The MDM2 (mouse double minute 2 homolog), also known as E3 ubiquitin protein ligase Mdm2, is an oncogene which encodes a Mdm2 protein, which is a key negative regulator of the p53 tumor suppressor, degrading p53 protein or inhibiting p53 activity.[21] Inhibition of tumor suppressor genes or insensitivity to antigrowth signals occurs in most of the tumors. Incipient cancer cells must evade these antiproliferative signals if they are to prosper.[11] The current work showed MDM2 already amplified in our minimally invasive case. The over expression of MDM2 has been also observed in a wide variety of human tumors, as sarcoma, leukemia, breast carcinoma, melanoma, and glioblastoma.[22]

WHSC1L1 (Wolf-Hirschhorn syndrome candidate gene-1) encodes a short protein containing one PWWP domain and is expressed in many tissues. The function of this encoded protein is unclear, but the presence of PWWP domain, a putative site for protein-protein interaction, suggests a regulatory role.[23] WHSC1L1 has already been identified as an oncogene and it is amplified and overexpressed in lung carcinoma.[24]

FGFRs (fibroblast growth factor receptors), encoded by four genes (FGFR1, FGFR2, FGFR3, and FGFR4), are associated with many biological processes such as organ development, cell proliferation and migration. Several studies have described a role of FGFRs in tumorigenesis due to the regulation of diverse tumorigenesis-related processes, including cell survival, proliferation, inflammation, metastasis and angiogenesis. FGFR1 amplification has been identified mainly in lung cancer.[25]

CDK4 (cyclin-dependent kinases 4) is directly involved in driving the cell cycle.[25] Amplification of CDK4 has been observed in several malignancies including glioma, breast cancer, lymphoma, melanoma, and sarcoma. Sometimes CDK4 is co-amplified with MDM2. The protein encoded by this gene is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression.[26]

LRIG (human leucine-rich repeats and immunoglobulin-like domains) gene family includes: LRIG1, LRIG2 and LRIG3. LRIG expression has proven to be of prognostic value in different types of human cancers, including breast cancer, early stage invasive squamous cervical cancer, cutaneous squamous cell carcinoma, oligodendroglioma, and astrocytoma. LRIF1 functions as a tumor suppressor gene, while little is known about the functions of LRIG2 and LRIG3.[27]

WIF1 is Wnt inhibitory factor 1 gene. The protein encoded by this gene functions to inhibit WNT proteins, which are extracellular signaling molecules that play a role in embryonic development. This gene acts as tumor suppressor gene, and has been found to be epigenetically silenced in various cancers.[28]

Conclusion

In conclusion, we identified unique genomic profiles of copy number alterations among three cases of CXPA from RPA, and differences can be explained due to histopathological subtypes and invasiveness degrees. However, recurrent gains at 3q and 8q, and amplifications at 12q14.3 and 12q15 here detected can be the promotional factors in the recurrence of the disease.

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Conflicts of interest

The authors declare no conflicts of interest.

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