Studies of genomic imbalances and the *MYB-NFIB* gene fusion in polymorphous low-grade adenocarcinoma of the head and neck

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Abstract. Polymorphous low-grade adenocarcinoma (PLGA) is a malignancy predominantly originating from the minor salivary glands. The molecular events underlying the pathogenesis of PLGA is poorly understood and no recurrent genetic aberrations have so far been identified. We used genome-wide, high-resolution aCGH analysis to explore genomic imbalances in 9 cases of PLGA. Because of the well-known morphologic similarities between PLGA and adenoid cystic carcinoma (ACC) we also analyzed all tumors for expression of the recently identified ACC-associated *MYB-NFIB* gene fusion. aCGH analysis revealed that the PLGA genome contains comparatively few copy number alterations (CNAs). Gains/losses of whole chromosomes or chromosome arms were more than twice as common as partial CNAs. Two cases showed gain of chromosome 8 and one case each gain of chromosome 9, loss of chromosome 22 and loss of the Y chromosome. One case showed loss of the entire 6q arm and one case an interstitial deletion of a 33-Mb segment within 6q22.1-q24.3. This region contains the *MYB* oncogene and the candidate tumor suppressor gene *PLAGL1*. RT-PCR analysis revealed that one of the 9 PLGAs expressed the ACC-associated *MYB-NFIB* gene fusion, illustrating the diagnostic difficulties associated with the diagnosis of these morphologically partly overlapping entities. Taken together, our findings indicate that the PLGA genome is genetically stable and contains comparatively few CNAs which is in line with the clinical observation that PLGA is a slow-growing, low-grade carcinoma with low metastatic potential.

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Introduction

Polymorphous low-grade adenocarcinoma (PLGA) is a neoplasm predominantly originating from the minor salivary glands. The term PLGA was introduced about twenty-five years ago and unified tumors described as terminal duct carcinoma, low-grade papillary adenocarcinoma and lobular carcinoma (1). Clinically, PLGA usually has an indolent course and distant metastases are uncommon at the time of diagnosis (2). PLGA are almost exclusively found in the oral cavity, particularly in the palate, upper lip and buccal mucosa (3). It is the second most common intraoral salivary gland carcinoma. The primary treatment is surgery with wide local resection. Although negative margins may be present in the surgical specimen, some tumors have a tendency to recur and/or metastasize many years after surgery, thus further emphasizing the need for radical resection with wide margins and long-term follow-up of the patients.

Histopathologically, PLGA may be a challenging diagnosis. The two main differential diagnoses are adenoid cystic carcinoma (ACC) and pleomorphic adenoma. PLGA is typically characterized by morphologic diversity, cytologic uniformity and an infiltrative growth pattern (3,4). A variety of growth patterns may be observed both between tumors and within individual tumors, including cribriform, lobular, trabecular, papillary, papillary-cystic or small duct-like structures. Mitotic figures are rare and necrosis is not a typical finding.

The molecular pathogenesis of PLGA is poorly understood. There are only a few cytogenetic and molecular genetic studies described in the literature. We have previously described two cases of intraoral PLGAs one of which showed monosomy 22 as the sole anomaly and the other a small clone with a reciprocal t(6;9)(p21;p22) translocation (5,6). Martins *et al* have described three cases of primary intraoral PLGA with translocations involving chromosome 12 with breakpoints at 12q13, 12q22 and 12p12.3 (7). In a previous study of the expression and mutation patterns of p53 in a series of 305 salivary gland neoplasms we found that PLGA had the highest prevalence of p53 expression (38%) of all salivary gland neoplasms (8). The corresponding figures for the differential diagnoses ACC and pleomorphic adenoma were 9 and 0%, respectively, suggesting that p53 may be a useful biomarker to help discriminate between these tumor types.

To learn more about the molecular genetics of PLGA and to identify possible recurrent aberrations in this tumor type we have now studied a series of 9 cases of PLGA using high-resolution array comparative genomic hybridization (aCGH). To further characterize the tumors we also studied the expression of the ACC-associated *MYB-NFIB* gene fusion (9).

Materials and methods

Tumor material. Fresh-frozen tumor tissue was available from two previously cytogenetically analyzed PLGAs (5,6). In addition, we had access to formalin-fixed paraffin-embedded (FFPE) tumor material from seven PLGAs from the Salivary Gland Tumor Registry at Albertinen Pathologie, Hamburg. All cases were primary lesions. The tumors were re-examined and the diagnosis of PLGA confirmed. Pertinent clinicopathological data of the tumors are shown in Table I.

Oligonucleotide aCGH analysis. Genomic DNA was isolated from frozen tumor tissue (cases 1-2) using the QIAamp® DNA mini kit (Qiagen GmbH, Hilden, Germany) and from FFPE tumor tissue (cases 3-9) using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) as described for FFPE material (Agilent Technologies Inc. Palo Alto, CA). aCGH analysis was subsequently performed using the Human Genome CGH Microarray 244K oligonucleotide arrays (G4411B sourced from the NCBI genome Build 35; Agilent Technologies). This array contains ~236,000 probes and has an average spatial resolution of ~6.4 kb. The experiments were performed essentially as recommended by the manufacturer (10,11). Slides were scanned on an Agilent High-Resolution C microarray scanner, followed by data extraction and normalization using Feature Extraction v.10.5 (Agilent Technologies) with linear normalization (protocol CGH-v4_95). Data analysis was carried out using the Genomic Workbench Standard Edition 5.0.14 software (Agilent Technologies). Detection of copy number gains and losses were based on the z-score algorithm (threshold 2.5) with a moving average of 2 MB and visual inspection of the log2 ratios. The thresholds of log2 ratio values for gain and loss were 0.3 and -0.3, respectively.

RT-PCR analysis. Total RNA was extracted from five 10-μm sections obtained from paraffin-blocks of seven PLGAs. The expression of *MYB-NFIB* fusion transcripts was subsequently studied by RT-PCR using *MYB*- and *NFIB*-specific primers as previously described (12,13). All tumors were screened for the most common *MYB-NFIB* fusion transcript variants, that is *MYB* exon 14 fused to *NFIB* exons 8a, 8c, or 9, respectively. In addition, tumors that were negative for these transcript variants were analyzed for expression of chimeric transcripts consisting of *MYB* exon 12 fused to *NFIB* exon 9. The *MYB* and *NFIB* exons were numbered as described elsewhere (9). As positive controls, ACCs with known *MYB-NFIB* fusion transcript variants were used (12,13).

Results

Genomic imbalances in PLGA. To determine the frequency and distribution of copy number alterations (CNAs) in PLGA we performed aCGH analysis of 9 tumors. All cases were located in the oral cavity, mainly in the soft and hard palate (six cases). The median age of the five female and four male patients was 68 years (mean age 61). Clinical follow-up data was only available from two cases. Case 1 had a local surgical excision followed by postoperative radiation therapy and there was no evidence of local recurrence or metastasis 17 years after the initial treatment. Case 2 was treated with a local radical excison and 9 years after surgery the patient showed no evidence of disease.

Table I. Clinicopathological data, copy number alterations and *MYB-NFIB* gene fusion status in 9 PLGAs.

Case no.	Sex/age (years)	Site	Copy number alterations: Losses	Copy number alterations: Gains	*MYB-NFIB* fusion status
1	M/17	Buccal mucosa	15q21.3 (290 kb; 4 genes) 22		-
2	M/65	Palate	Y		-
3	M/70	Palate	6q	8	-
4	M/65	Palate			-
5	F/71	Palate	6q22.1-q24.3 (33 Mb; 151 genes)		-
6	F/68	Gingiva			-
7	F/42	Palate			+
8	F/70	Lip			-
9	F/83	Palate		8, 9	-

aCGH analysis of the 9 PLGAs revealed a total of 8 genomic imbalances (five losses and three gains) in five tumors. The remaining four tumors (cases 4 and 6-8) had no CNAs. Gene amplifications and homozygous deletions were not detected in any of the tumors. A detailed description of all copy number gains and losses are shown in Table I. Gains/losses of whole chromosomes or chromosome arms were more than twice as common as partial genomic imbalances. Two cases showed gain of one chromosome 8 (Fig. 1A) and one case each gain of chromosome 9, loss of chromosome 22 and loss of the Y chromosome. One case had loss of the entire 6q arm and one case an interstitial deletion of a 33-Mb segment within 6q22.1-q24.3 (Fig. 1B). This region contains 151 known genes including the *MYB* oncogene and the candidate tumor suppressor gene *PLAGL1*. We also detected one case with loss of a 290-kb segment within 15q21.3. This region only contains the four known genes *PIGB*, *CCPG1*, *DYX1C1* and *PYGO1*.

Expression of MYB-NFIB fusion transcripts in PLGA. Because of the well-known morphologic similarities between PLGA and ACC we also screened our 9 PLGAs for expression of the ACC-associated gene fusion *MYB-NFIB*. RT-PCR analysis of cDNAs prepared from the 9 tumors revealed that all cases except one were *MYB-NFIB* fusion-negative (Fig. 2). In case 7, we found expression of a 232-bp chimeric *MYB-NFIB*

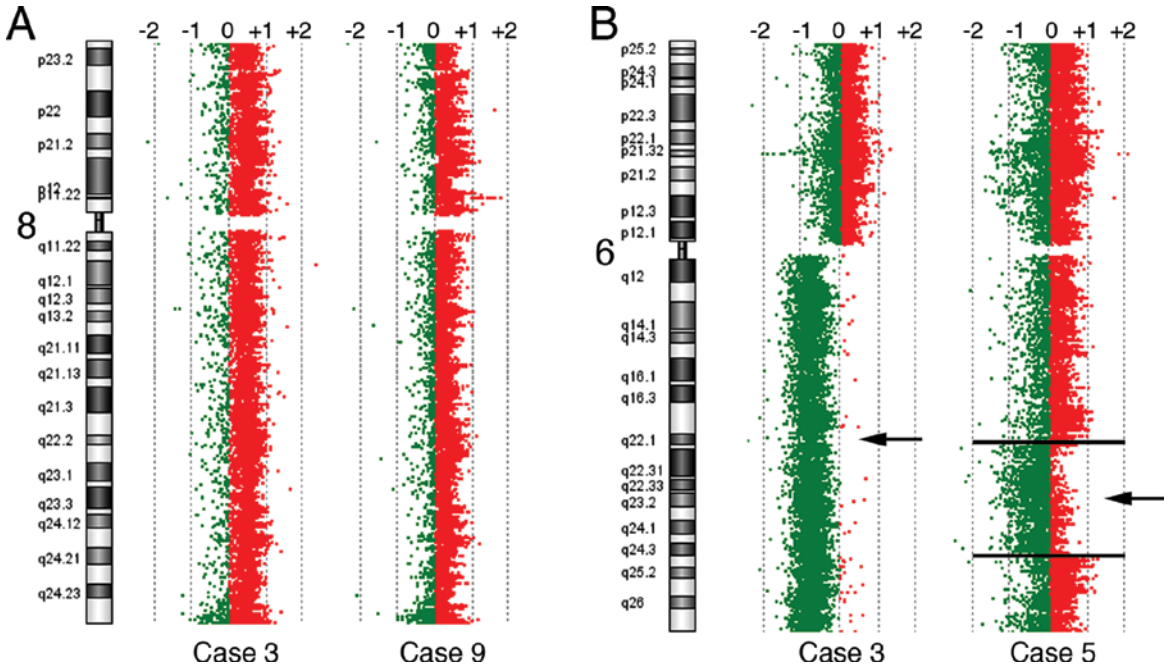

Figure 1. aCGH profiles showing (A) gain of an entire chromosome 8 in cases 3 and 9, and (B) loss of an entire 6q arm (case 3) and segmental loss of 6q22-1-q24.3 (case 5). Red dots indicate gains and green dots indicate losses.

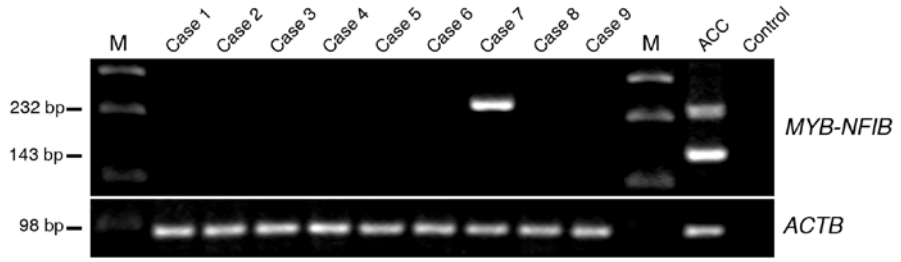

Figure 2. Expression of *MYB-NFIB* fusion transcripts in PLGA. RT-PCR analysis of the 9 PLGAs using primers located in *MYB* exon 14 and *NFIB* exon 9. Size markers (M), positive control (fusion-positive ACC) and negative control (H_2O). *ACTB* was used as internal control to test for intact RNA and cDNA.

transcript consistent with a fusion of *MYB* exon 14 to *NFIB* exon 8c.

Discussion

The molecular events underlying the pathogenesis of PLGA is poorly understood and no recurrent genetic aberrations have so far been identified. Here we have used genome-wide, high-resolution aCGH analysis to explore genomic imbalances in 9 cases of PLGA. This is to our knowledge the first study employing high-resolution aCGH to identify genetic alterations in PLGA.

The most striking observation in the present series of PLGAs is perhaps the lack of CNAs or very few CNAs detected in these low-grade, invasive carcinomas. Four tumors had no detectable CNAs, three cases had two CNAs each, and one case had a single copy number loss. In addition, we did not observe amplifications or homozygous deletions in any of the tumors. Although the number of tumors analyzed is limited, our findings suggest that CNAs are not likely to be of significant importance for the genesis and/or progression of PLGA. However, we cannot exclude the possibility that we might have missed a few CNAs in the DNAs isolated from FFPE tissue (cases 3-9) since this type of material is known to generate DNA of inferior quality compared to DNA obtained from fresh-frozen tumor tissue. Taken together, our findings indicate that the PLGA genome is genetically stable and contains comparatively few CNAs. This is in line with the fact that PLGA is a slow-growing, low-grade neoplasm with low metastatic potential. Interestingly, we have recently found that also low-grade ACCs and mucoepidermoid carcinomas have few or no CNAs (Persson *et al*; unpublished observations). Both these tumor types are characterized by recurrent chromosome translocations resulting in potent oncogenic gene fusions, that is *MYB-NFIB* fusions in ACC and *CRTC1-MAML2* gene fusions in mucoepidermoid carcinoma (9,14-16). Whether also PLGA may be characterized by an oncogenic gene fusion is currently not known. Previous cytogenetic analyses do, however, suggest that this is not the case since no recurrent translocations/rearrangements have been found in PLGA.

Because of the well-known morphologic similarities between PLGA and ACC (4) we screened our 9 PLGAs for expression

of the *MYB-NFIB* gene fusion. Much to our surprise we found that one of the tumors was fusion-positive. This observation demonstrates the diagnostic difficulties associated with the diagnosis of these morphologically partly overlapping entities. Microscopic re-examination of the fusion-positive case did, however, not change the original morphologic diagnosis of PLGA. However, since the *MYB-NFIB* fusion has not been detected in any other salivary gland tumor (including PLGA) than ACC (9,12,17,18) we believe that this case may represent a low-grade variant of ACC that mimics PLGA. Taken together, these findings further highlight the utility of the *MYB-NFIB* fusion as a diagnostic biomarker.

Cases 1 and 2 have previously been cytogenetically characterized (5,6). Using aCGH we could confirm the original observations of loss of chromosome 22 in case 1 and loss of the Y chromosome in case 2. In addition, aCGH revealed a small interstitial deletion of a 290-kb segment within 15q21.3 in case 1. This region only contains four known genes, *PIGB*, *CCPG1*, *DYX1C1* and *PYGO1*. *PIGB* encodes a transmembrane protein located in the endoplasmic reticulum and is involved in GPI-anchor (glycosylphosphatidylinositol) biosynthesis, *CCPG1* encodes a protein that acts as an assembly platform for Rho-protein signaling complexes and is involved in cell cycle regulation (19), *DYX1C1* is involved in a chromosomal translocation associated with susceptibility to developmental dyslexia (20), and *PYGO1* encodes a protein involved in signal transduction through the Wnt signaling pathway (21). Whether loss of one or more of these genes may contribute to PLGA tumorigenesis remains, however, to be shown.

The only recurrent CNAs found in the present series of PLGAs were gain of chromosome 8 and complete or partial loss of 6q found in two cases each. Gain of chromosome 8 has previously been found in subsets of pleomorphic adenomas, carcinoma ex pleomorphic adenomas, mucoepidermoid carcinomas and acinic cell carcinomas (11,22,23). In pleomorphic adenomas and its malignant counterpart, *PLAG1* (pleomorphic adenoma gene 1) has been suggested as the most likely target gene (11). Similarly, deletions involving 6q have previously been found in all major histologic subtypes of salivary gland carcinomas (23-25, Persson *et al*; unpublished observations). In case 3 there was a deletion of the entire 6q arm whereas in case 5 there was an interstitial deletion of 6q22.31-6q24.3. Using low-resolution chromosomal-based CGH, Hannen and co-workers have previously described a case of PLGA with a terminal deletion of 6q23-qter (26), thus further underscoring the significance of loss of 6q in PLGA. There are 151 known genes located within the deleted 6q22.31-q24.3 segment in case 5, including the MYB oncogene and the candidate tumor suppressor gene *PLAGL1*. Other potentially interesting genes in this region are *DSE* (tumor rejection antigen), *ASF1A* (efficient senescence-associated cell cycle exit), *BCLAF1* (death-promoting transcriptional repressor) and *PERP* (TP53 apoptosis effector). It remains to be shown whether any of these genes are the actual targets of the 6q deletions in PLGA.

In summary, our findings indicate that the PLGA genome is genetically stable and contains few CNAs in line with the relatively benign biological behavior of this low-grade carcinoma. PLGAs also differ cytogenetically and molecularly from pleomorphic adenomas and ACCs which are the main differential diagnoses. The latter tumors are characterized by recurrent chromosome rearrangements resulting in gene fusions involving the *PLAG1*, *HMGA2* and *MYB* oncogenes (16, 22). Whether also PLGA may be characterized by a tumor-type specific gene fusion remains to be shown.

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References

1. Evans HL and Batsakis JG: Polymorphous low-grade adenocarcinoma of minor salivary glands. A study of 14 cases of a distinctive neoplasm. Cancer 53: 935-942, 1984.
2. Pogodzinski MS, Sabri AN, Lewis JE and Olsen KD: Retrospective study and review of polymorphous low-grade adenocarcinoma. Laryngoscope 116: 2145-2149, 2006.
3. Paleri V, Robinson M and Bradley P: Polymorphous low-grade adenocarcinoma of the head and neck. Curr Opin Otolaryngol Head Neck Surg 16: 163-169, 2008.
4. Luna MA and Wenig BM: Polymorphous low-grade adenocarcinoma. In: World Health Organization Classification of Tumors. Pathology and Genetics Head and Neck Tumours. Barnes L, Eveson J, Reichart P and Sidransky D (eds.) IARC Press, Lyon, pp223-224, 2005.
5. Mark J, Dahlenfors R, Stenman G, Bende M and Melen I: Cytogenetical observations in two cases of polymorphous low-grade adenocarcinoma of the salivary glands. Anticancer Res 12: 1195-1198, 1992.
6. Dahlenfors R, Gertzen H, Wedell B and Mark J: Cytogenetical observations in a cultured polymorphous low-grade adenocarcinoma originating from the minor salivary glands. Anticancer Res 17: 105-106, 1997.
7. Martins C, Fonseca I, Roque L, Ribeiro C and Soares J: Cytogenetic similarities between two types of salivary gland carcinomas: adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma. Cancer Genet Cytogenet 128: 130-136, 2001.
8. Nordkvist A, Röijer E, Bang G, Gustafsson H, Behrendt M, Ryd W, Thoresen S, Donath K and Stenman G: Expression and mutation patterns of p53 in benign and malignant salivary gland tumors. Int J Oncol 16: 477-483, 2000.
9. Persson M, Andrén Y, Mark J, Horlings HM, Persson F and Stenman G: Recurrent fusion of MYB and NFIB transcription factor genes in carcinomas of the breast and head and neck. Proc Natl Acad Sci USA 106: 18740-18744, 2009.
10. Barrett MT, Scheffer A, Ben-Dor A, Sampas N, Lipson D, Kincaid R, Tsang P, Curry B, Baird K, Meltzer PS, Yakhini Z, Bruhn L and Laderman S: Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. Proc Natl Acad Sci USA 101: 17765-17770, 2004.
11. Persson F, Winnes M, Andrén Y, Wedell B, Dahlenfors R, Asp J, Mark J, Enlund F and Stenman G: High-resolution array CGH analysis of salivary gland tumors reveals fusion and amplification of the FGFR1 and PLAG1 genes in ring chromosomes. Oncogene 27: 3072-3080, 2008.
12. Brill LB, 2nd, Kanner WA, Fehr A, Andrén Y, Moskaluk CA, Löning T, Stenman G and Frierson HF Jr: Analysis of MYB expression and MYB-NFIB gene fusions in adenoid cystic carcinoma and other salivary neoplasms. Mod Pathol: May 13, 2011 (Epub ahead of print).
13. Fehr A, Kovács A, Löning T, Frierson HF, van den Oord JJ and Stenman G: The MYB-NFIB gene fusion - a novel genetic link between adenoid cystic carcinoma and dermal cylindroma. J Pathol 224: 322-327, 2011.
14. Tonon G, Modi S, Wu L, Kubo A, Coxon AB, Komiya T, O'Neil K, Stover K, El-Naggar A, Griffin JD, Kirsch IR and Kaye FJ: t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. Nat Genet 33: 208-213, 2003.

15. Enlund F, Behboudi A, Andrén Y, Öberg C, Lendahl U, Mark J and Stenman G: Altered Notch signaling resulting from expression of a WAMTP1-MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. Exp Cell Res 292: 21-28, 2004.
16. Stenman G, Andersson MK and Andrén Y: New tricks from an old oncogene: gene fusion and copy number alterations of MYB in human cancer. Cell Cycle 9: 2986-2995, 2010.
17. Mitani Y, Li J, Rao PH, Zhao YJ, Bell D, Lippman SM, Weber RS, Caulin C and El-Naggar AK: Comprehensive analysis of the MYB-NFIB gene fusion in salivary adenoid cystic carcinoma: incidence, variability and clinicopathological significance. Clin Cancer Res 16: 4722-4731, 2010.
18. West RB, Kong C, Clarke N, Gilks T, Lipsick JS, Cao H, Kwok S, Montgomery KD, Varma S and Le QT: MYB expression and translocation in adenoid cystic carcinomas and other salivary gland tumors with clinicopathologic correlation. Am J Surg Pathol 35: 92-99, 2011.
19. Kostenko EV, Olabisi OO, Sahay S, Rodriguez PL and Whitehead IP: Ccpg1, a novel scaffold protein that regulates the activity of the Rho guanine nucleotide exchange factor Dbs. Mol Cell Biol 26: 8964-8975, 2006.
20. Taipale M, Kaminen N, Nopola-Hemmi J, Haltia T, Myllyluoma B, Lyytinen H, Muller K, Kaaranen M, Lindsberg PJ, Hannula-Jouppi K and Kere J: A candidate gene for developmental dyslexia encodes a nuclear tetratricopeptide repeat domain protein dynamically regulated in brain. Proc Natl Acad Sci USA 100: 11553-11558, 2003.
21. Thompson B, Townsley F, Rosin-Arbesfeld R, Musisi H and Bienz M: A new nuclear component of the Wnt signalling pathway. Nat Cell Biol 4: 367-373, 2002.
22. Stenman G: Fusion oncogenes and tumor type specificity-insights from salivary gland tumors. Semin Cancer Biol 15: 224-235, 2005.
23. Mitelman F, Johansson B and Mertens F (eds.): Mitelman database of chromosome aberrations and gene fusions in cancer. http://cgap.nci.nih.gov/Chromosomes/Mitelman, 2011.
24. Sandros J, Stenman G and Mark J: Cytogenetic and molecular observations in human and experimental salivary gland tumors. Cancer Genet Cytogenet 44: 153-167, 1990.
25. Enlund F, Persson F and Stenman G: Molecular analyses of the candidate tumor suppressor gene, PLAGL1, in benign and malignant salivary gland tumors. Eur J Oral Sci 112: 545-547, 2004.
26. Hannen EJ, Bulten J, Festen J, Wienk SM and de Wilde PC: Polymorphous low grade adenocarcinoma with distant metastases and deletions on chromosome 6q23-qter and 11q23-qter: a case report. J Clin Pathol 53: 942-945, 2000.