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 recently identified ACC-associated MYB-NFIB gene fusion. 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 cyogenetically 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 excision and 9 years after surgery the patient showed no evidence of disease.

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, DYPIC1 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

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

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex/age</th>
<th>Site</th>
<th>Copy number alterations</th>
<th>MYB-NFIB fusion status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/17</td>
<td>Buccal mucosa</td>
<td>15q21.3 (290 kb; 4 genes)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>M/65</td>
<td>Palate</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>M/70</td>
<td>Palate</td>
<td>6q 8</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>M/65</td>
<td>Palate</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>F/71</td>
<td>Palate</td>
<td>6q22.1-q24.3 (33 Mb; 151 genes)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>F/68</td>
<td>Gingiva</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>F/42</td>
<td>Palate</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>F/70</td>
<td>Lip</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>F/83</td>
<td>Palate</td>
<td>8, 9</td>
<td>-</td>
</tr>
</tbody>
</table>
transcript consistent with a fusion of \textit{MYB} exon 14 to \textit{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 \textit{MYB-NFIB} fusions in ACC and \textit{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

![Figure 1](image1.png)

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](image2.png)

Figure 2. Expression of \textit{MYB-NFIB} fusion transcripts in PLGA. RT-PCR analysis of the 9 PLGAs using primers located in \textit{MYB} exon 14 and \textit{NFIB} exon 9. Size markers (M), positive control (fusion-positive ACC) and negative control (H2O). \textit{ACTB} was used as internal control to test for intact RNA and cDNA.
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, CCGP1, DXYC1 and PYGO1. PIGB encodes a transmembrane protein located in the endoplasmic reticulum and is involved in GPI-anchor (glycosylphosphatidylinositol) biosynthesis, CCGP1 encodes a protein that acts as an assembly platform for Rho-protein signaling complexes and is involved in cell cycle regulation (19), DXYC1 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 PLAG1. Other potentially interesting genes in this region are DSE (tumor rejection antigen), ASFI4 (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


