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Genomic changes in salivary gland pleomorphic adenomas detected by comparative genomic hybridization

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The aim of this study was to investigate whether so far unknown chromosomal alterations in pleomorphic adenoma (PA) exist. To this end, tissue samples from 18 patients with parotid gland PA were studied by comparative genomic hybridization (CGH) using Phi-29-DNA-polymerase for DNA amplification. The most common aberrations were losses of chromosomal material of 19p (6/18), 9q, 16p, and 19q (in 3 out of 18 patients each). Additional losses were observed on 4p, 5q, and 17q (2 / 18 each). Gains involved chromosomes 2p, 4p, 6p, 17q, and 21q (2 / 18 each). Losses of 19p have been associated with inactivation of tumor-suppressor genes in carcinomas previously. As a result, pleomorphic adenomas show a considerable diversity of chromosomal gains and losses detected by CGH. The 19p arm, and particularly its 19p13 region, need be further studied to elucidate the potential impact of associated lost tumor suppressor genes on PA development.

Key words: pleomorphic adenoma, comparative genomic hybridisation, salivary gland, parotid, chromosomal

Pleomorphic adenomas are the most common tumors of the major salivary glands. Although they are benign tumors, they may undergo malignant transformation. From the pathologist's point of view, they are biphasic tumors, comprised of ductal epithelial and myoepithelial cells. The proportions of myoepithelial and epithelial components exhibit considerable variance. More often than not the myoepithelial component predominates. The epithelial component exhibits variance in the degree and type of differentiation and metaplasia.

Karyotypic alterations of parotid pleomorphic adenomas (PAs) have been studied since 1973 [1]. Initially, three cytogenetically different groups of pleomorphic adenomas were differentiated. One group, mostly composed by younger patients, showed alterations in the 8q12 region. A second group, which included mainly older patients, showed alterations at the 12q13-15 region and in a third group no alterations of genetic material could be disclosed [2]. The detected alterations, principally concerning chromosomes 8 and 12, were translocations. The most well-known and best studied translocation is the one between 8q12 and 3p21. On 8q12 is located the PLAG1 (pleomorphic adenoma gene 1) gene, which plays a central role in cancerogenesis of PA. On 3p21 is located the CTNNB1 gene which encodes ß-catenin, a protein important for intercellular contacts [3]. By this partial gene exchange the promoter activity of each gene is altered, in such a manner that PLAG1 expression increases while ß-catenin expression decreases [4]. Another PLAG1-translocation to chromosome 5 was discovered, which also caused an increased PLAG1 expression. The translocation partner on chromosome 5 was the LIFR gene, which encodes a receptor for a substance belonging to the interleukin 6 group [5,6]. Translocations are not always associated with detectable karyotypic changes. For instance, a fusion of PLAG1 with S II (TCEAI) was not accompanied by any visible alteration [7]. The S II (TCEAI) gene, which is located at the 3p21.3-22 position [8] encodes a RNA-polymerase II and hence plays an important role in the regulation of transcription mechanisms [9]. Fusions without karyotypic changes on the same arm of chromosome 8 have been reported. The CHCHD7 gene, whose function is not yet definitely unraveled, is located just 500 bp away from PLAG1 and is fused with PLAG1 in a number of PA tumors. Through this fusion a change in the promoter region and a respective over-expression of PLAG1 results [10]. Through the aforementioned translocations the changes in the promoter region result in an increased transcription of PLAG1. The peptide that is encoded by PLAG1 is a transcription factor that binds to the IGF2 promoter region increasing the gene's rate of transcrip-

Abbreviations: PA – Pleomorphic Adenoma; CGH – comparative genomic hybridisation

tion. IGF2 stimulates cell proliferation and cell growth and hence promotes tumor development [11]. At least another 12 genes whose expression is regulated by PLAG1 in PAs have been detected. Among them are the gene encoding the growth factor pirin, genes encoding peptides involved in metabolism and other genes whose role is not yet discovered [12].

Comparative Genomic Hybridization (CGH) offers the opportunity to analyze gains and losses of chromosomal material by the hybridisation of normal genomic DNA with, e.g., whole DNA extracted from tumors. In the first published study of parotid PAs by means of CGH [13] the aforementioned regions had neither shown gains nor losses. However, gains had been discovered in the 20p12.1 region and losses in the 9q12-21.11, 16q11.2 and 5q12.4-14.1 regions. Giefing et al. [14] studied PAs by CGH and found, in accordance with Toida et al. [13], no alterations of the 8q region, but detected alterations at the 12q24.2, 17p13.1 and 13q22.1-22.2 loci. These results concerning the PLAG-locus are not astonishing as small mutations or balanced translocations are not detectable by the CGH method.

The aim of the present study was to investigate by CGH whether so far unknown chromosomal alterations exist, as the multiple mechanisms and pathways resulting in the heterogeneous PA growth are not as yet fully understood.

Patients and methods

Tissue samples.Tissue specimens were taken intra-operatively from 18 patients (12 female, 6 male; age range 21 – 82 years) with histologically confirmed pleomorphic adenoma of the parotid gland (table 1). The pleomorphic adenoma specimens comprised 14 frozen specimens and 4 paraffin – embedded specimens.

Tissue specimens of PAs were put into liquid nitrogen immediately after removal of the tumors and stored until further use at -80°C or were fixed in formalin and the resulting paraffin blocks were than stored at room temperature.

This study was approved by the institutional review board and performed in accordance to the actual version of the declaration of Helsinki. Informed consent was obtained. All patients were operated between 1993 and 2005 in our department.

Comparative genomic hybridization (CGH). The DNA-isolation procedure, DNA-labeling and the CGH-protocol have been described in detail elsewhere [15]. In brief, normal control DNA was isolated from peripheral blood lymphocytes. To isolate DNA from paraffin – embedded tissues, 7 µm – sections were cut; from kryo-preserved tissues 14 µm sections were cut. DNA was prepared by digestion with Proteinase K (Roche), and stored at -20°C until later use. For amplification of the isolated DNA we utilized the Phi-29-DNA-polymerase using a Genomiphi DNA amplification kit (Amersham). Labeling of tumor and control DNA with Biotin-11-dUTP and Digoxygenin-11-dUTP, respectively, was than performed using a nick translation kit (Roche).

Table 1. Demographic data and observed chromosomal losses and gains of the analysed pleomorphic adenoma specimens (n.a. = not available - = no aberration observed)

patient	age	gender	size (max. diameter [cm])	losses	gains
1	34	M	1.1	4q34-35.1 6q16.1-16.3	8q11.21-21.3 20q11.22-13.11
2	55	F	6	13q33.1-ter	1q21.1-21.3 2p11.1-13.1 2q37.1-ter 4p16.2-ter 7p22-ter
3	55	F	2.7	-	1p35.1-36.12 6p25.1-ter 15q11.1-13.1 16p12.2-13.12 17p13.2-ter 20p13-ter
4	30	F	3	4p16.2-ter 9q34.11-34.2 16p13.2-ter 17p13-1-ter 17q24.1-25.1 19p11-ter 19q13.2-13.41 20q13.2-13.33 21q22.12-22.2	2p25.1-ter 2q22.1-23.1 3p26.1-ter 6p25.1-ter 9p24.1-ter
5	65	F	5.5	1p36.3-ter 9q34.12-34.2 16p13.3-ter 17q24.3-25.1 19p13.12-ter 22q13.1-13.3	9p13.3-23
6	56	F	4	4p16.3-ter 9q34.12-34.3 19p13.2-ter 19p13.11-13.12	-
7	21	F	3.2	-	-
8	22	F	2.2	-	-
9	33	F	1.2	16p13.3-ter	4p16.2-ter
10	82	F	2.6	3q21.1-24 19p13.3-ter	-
11	32	F	1.5	5q13.3-35.1 12p13.2-ter	-
12	58	F	3.5	-	-
13	56	M	3	5q13.1-35.1 19p13.11-ter 19q13.33-13.41	1q22-43
14	64	M	4.5	19p12-ter 19q13.2-13.41	-
15	70	M	n.a.	18p11.32-ter	-
16	58	M	n.a.	-	2p25.2-ter 11p15.4-ter 17q25.1-ter
17	60	F	n.a.	-	17q24.3-ter 21q22.12-ter
18	n.a.	M	n.a.	-	9q34.12-34.2 19p13.3-ter 21q22.2-ter

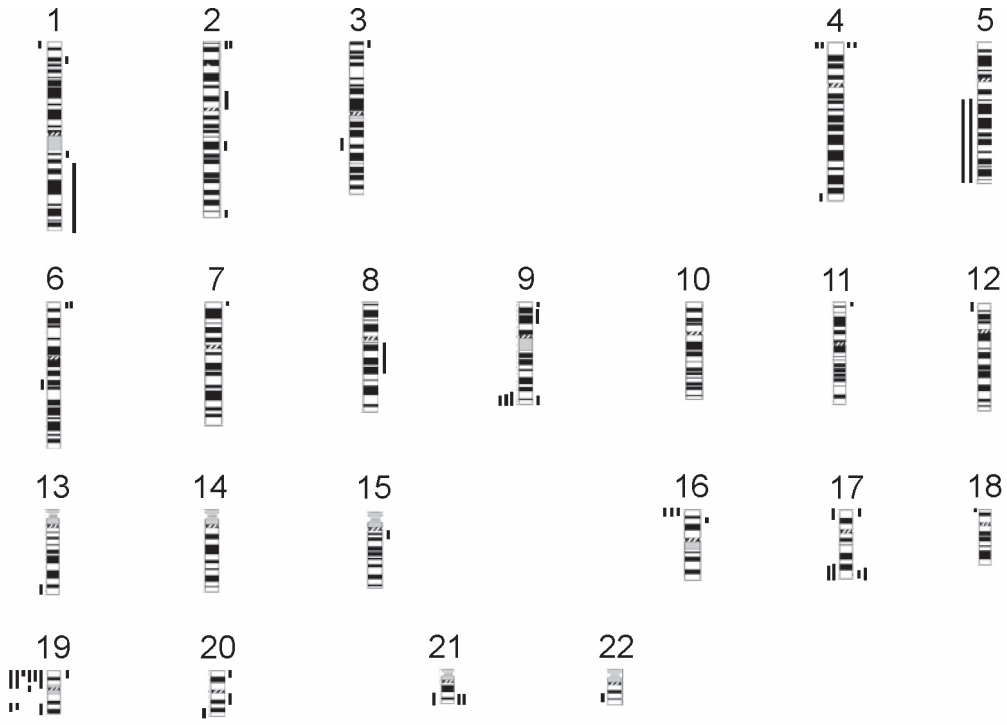

Figure 1. Gains and losses of chromosomal material detected in pleomorphic adenoma specimens. Gains are depicted on the right side and losses on the left side of each chromosome.

For hybridization 1 μg each of Digoxygenin and Biotin labeled probe and control DNA were co-precipitated with 70 μg of Cot1-DNA (Gibco BRL Life Technologies). Precipitated DNA was re-suspended in 5 μl of deionized formamide and incubated for 1 hour at room temperature. Hybridization buffer (5 μl: 4x SSC, 20 % dextransulfate in a sodium phosphate buffer (pH 7.0)) was added, incubated for additionally 30 min, denaturated for 5 min at 75°C, and then directly transferred to 37°C for 30 min in a moist chamber for pre-annealing.

Prior to hybridization, commercially available metaphase chromosomes (Abbott) were denatured. The hybridization mixture was then applied to the pre-warmed slides (42°C), covered and sealed with rubber cement. The hybridization reaction was performed in a moist chamber at 37°C for at least 36 hours. The slides were than stained with anti-Digoxygenin-MAb-Fab-Rhodamine and Streptavidine-FITC (Roche), and counterstained with DAPI (Vector Laboratories).

For analysis a cooled CCD camera, coupled to a Zeiss Axioskope epifluorescence microscope (Carl Zeiss, Jena, Germany) and linked to appropriate hard- and software was used. The superimposed and pseudocolored images were acquired sequentially using bandpass filter sets appropriate for DAPI, FITC and Rhodamin, respectively.

Data from at least 10 metaphases were averaged in each case. Fluorescence ratios <0.75 were considered as losses of chromosomal material, ratios >1.25 as overrepresentations (gains) [16].

Due to repetitive DNA-elements and resulting unspecific hybridisations of the gonosomes, gains and losses on chromosomes X- and Y have not been analysed in this study.

Statistics. Data were analyzed and descriptively reported. Due to the low tumor sample numbers available no formal statistics were calculated.

Results

Size of the pleomorphic adenoma tumors. The studied tumors' average size was 3,1 cm. The largest tumor was 6 cm in max. diameter and the smallest was 1,1 cm (table 1). The tumor with the greatest number of gains and losses (patient no. 4; 5 gains, 9 losses) was 3 cm in max. diameter. On the contrary, the largest (6 cm max. diameter; patient no. 2) tumor showed only one loss and 5 gains. The smallest tumor (patient no. 1) had 2 gains and two losses. There was no apparent correlation between the tumor size and the number of gains and losses.

Age of the pleomorphic adenoma patients. Patients' age were ranging between 21 and 82 (mean: 50.1) years. There was no correlation between patients' age and the number of gains or losses. The youngest patient, a 21-year-old female, showed no changes on autosomal chromosomes, while the oldest patient, a 82-year-old female, had two losses. The greatest cumulative number of gains and losses was discovered in a 30-year-old female (patient no. 4) in whom a total of 14 gains and losses were detected.

Chromosomal aberrations in pleomorphic adenomas. In 4 out of 18 specimens only gains (without any losses) were discovered, while in 5 out of 18 specimens only losses (without any gains) were found. In 6 specimens both gains and losses were discovered. In the remaining 3 specimens neither gains nor losses were detected (table 1 and figure 1). The most frequently recurring losses were found on chromosomes 19p (6/18), 9q (3/18), 16p (3/18), 19q (3/18), 4p (2/18), 5q (2/18), and 17q (2/18). Both losses on chromosome 5 were the largest seen in this series; they were extending over almost the entire length of the q-arm. A large gain was discovered on chromosome 1q (1/18). This gain was a high-level amplification and extended over almost the entire chromosomal arm. Less extensive but recurrent gains were observed on chromosomes 2p, 4p, 6p, 17q, and 21q (2/18 each). Additionally, several single, non-recurrent gains and losses were observed involving almost all chromosomes.

Genes located on chromosomal regions with recurring aberrations We observed in at least three patients recurrent losses of genetic material on four chromosomal regions, namely on chromosomes 9q in three patients, 16p (3 patients) 19p (6 patients), and on 19q (3 patients). Losses suggest the involvement of tumour suppressor genes (TSGs). Therefore, we investigated whether these chromosomal areas harboured genes potentially associated with the mechanisms of cell growth or tumour development (table 2). Additionally, we analysed chromosome 5q, as we observed in two patients losses of the entire long arm. Data on potential or known TSGs were extracted from the NCBI website (National Centre for Biotechnology Information; www.ncbi.nlm.nih.gov/gene).

Discussion

PLAG1 activation has been described as the main genetic association to pleomorphic adenoma. Mainly chromosomal translocations have been associated with PLAG1 activation. However, in earlier studies it was shown that not all PAs exhibit the aforementioned karyotypic alterations [2]. Out of 220 studied PA specimens more than 50% showed a normal karyotype, while 25.5% showed alterations at the 8q12 region and 13.2 % showed alterations at the 12q13-15 region. As mentioned before, several other genetic changes except translocations might be potentially involved, underlying the known clinically observed heterogenic growth patterns of PA. We therefore analyzed 18 PAs by CGH enabling the analysis of gains and losses of the whole genome. We found evidence for recurrent losses of partially so far unknown loci and identified genes that could play a role in the process of PA tumour development.

In two PA specimens a loss was detected on the long arm of chromosome 5 (5q13.1-35.3). This is in accordance with data reported by Toida et al. [13]. Accordingly, we identified two potential TSGs, namely EGR1 and FAT2. The EGR1 (early growth response 1) gene is a nuclear protein supposed to be a tumor suppressor gene. Decreased activity of the EGR1 gene has already been associated with various experimental tumors of the mammary and breast [17].

Table 2. Recurrent aberrations and genes potentially associated with pleomorphic adenoma. Shown are aberrations of chromosomal regions occurring in three or more samples and additionally the two samples with complete losses of the entire chromosomal arm 5q. Remarkably, only losses recured at the same loci in more than two tumours.

loss of chromosomal region	patient no	candidate gene (GeneID)
5q13.3-35.1	11	EGR1 = early growth response 1 (1958)
5q13.1-35.1	13	FAT2 = FAT tumor suppressor homolog 2 (2196)
9q34.11-34.2	4	PPL = Periplakin (5493)
9q34.12-34.2	5	DNAJA3 = DnaJ (Hsp40) homolog, subfamily A, member 3 (9093)
9q34.12-34.3	6	NTHL1 = nth endonuclease III-like 1 (4913) AXIN1 = axin 1 (8312)
16p13.2-ter	4	TSC1 = tuberous sclerosis 1 (7248)
16p13.3-ter	5	NOTCH1 = notch 1 (4851)
16p13.3-ter	9	
19p11-ter	4	LKB1 = serine/threonine kinase 11 (6794)
19p13.12-ter	5	BRG1 = SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (6597)
19p13.11-13.12	6	ZIPK = death-associated protein kinase 3 (1613)
19p13.2-ter		SAFB1 = scaffold attachment factor B1 (6294)
19p13.3-ter	10	SAFB2 = scaffold attachment factor B2 (9667)
19p13.11-ter	13	
19p12-ter	14	
19q13.2-13.41	4	GLTSCR1 = glioma tumor suppressor candidate region gene 1(29998)
19q13.33-13.41	13	GLTSCR2/PICT-1 = glioma tumor suppressor candidate region gene 2 (29997)
19q13.2-13.41	14	

At the same chromosomal region another tumor-suppressor gene, FAT2, is located [18]. FAT2 is a human homolog of the Drosophila fat gene, which encodes a protein essential for controlling cell proliferation during development. The gene product is a member of the cadherin superfamily. This protein most likely functions as a cell adhesion molecule, controlling cell proliferation.

Additionally, we observed losses at 9q in three patients. However, in one sample gains of genomic material occurred at the same region. Therefore, we hypothesized that at this region tumor growth promoting and tumor suppressing genes might be co-localized. Accordingly, we identified NOTCH1 and TSC. The Notch signalling pathway plays an important role in cellular differentiation, proliferation and apoptosis. Activating mutations in NOTCH1 have been identified in over 50% of T-cell acute lymphoblastic leukemias and NOTCH-targeting therapies are under development [19]. In ovarian cancer cells the depletion of NOTCH 1 led to growth inhibition [20].

Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome in which hamartomas develop in multiple organ systems. The TSC1-protein specifically suppresses the TOR complex 1 and the dysregulation of the mTOR signalling pathway has been implicated in the development of several cancers. Accordingly, the down-regulation of TSC1 has been shown in oral squamous cell carcinoma [21] and TSC1 loss synergizes with the Kras mutation to enhance lung tumorigenesis [22].

We observed deletions at the telomeric end of 16p in three cases. Several potential negative growth regulators are localized at this chromosomal region. Periplakin downregulation has been associated with esophageal squamous cell carcinoma [23]. Plakins were first identified in epithelial cells where they were found to connect the intermediate filaments to desmosomes and hemidesmosomes [24]. DNAJA3 is a human homologue of the Drosophila tumor suppressor protein Tid56, and its involvement in multiple growth regulating mechanisms such as apoptosis, cell proliferation, and cell survival has been advocated [25]. Its expression is related to colorectal cancer progression [26] and its function as a tumor suppressor has been shown in head and neck squamous cell carcinoma [27]. NTHL1 is a base excision repair enzyme capable of removing oxidized pyrimidines. Down-regulation of the gene has been associated with the pathogenesis of gastric cancer [28]. The down-regulation of AXIN1, a component of the Wnt-signalling cascade, has been observed in brain tumors, esophageal squamous cell carcinoma, lung cancer and others [29;30,31]. This potential tumor suppressor protein induces apoptosis and reduces proliferation by the activation of the p53 pathway [32].

On the telomeric region of chromosome 19p we found in 6 out of the analyzed 18 cases losses of chromosomal material, suggesting the loss of one or more tumor suppressor genes located in this region. GenBank-analysis revealed that losses at the 19p arm have been reported in other carcinomas as well and have been associated with a down-regulation of well-known or putative tumor-suppressor genes (TSGs). Losses of heterozygosity (LOH) of the short arm of chromosome 19 are frequent in lung cancer. The LKB1 gene (also called STK11; located at 19p13.3) is somatically inactivated through point mutations and large deletions in lung tumours. It is generally agreed that this alteration strongly predominates in non-small cell lung cancer, in particular adenocarcinomas, in smokers. Similar to LKB1, the BRG1 gene (also known as SMARCA4; located at 19p13.2) is somatically inactivated by point mutations or large deletions in lung tumors featuring LOH of chromosome 19p [33]. Deletion of 19p13 is one of the most frequent genetic changes in gastric carcinoma and is associated with down-regulation of the TSG encoding ZIP kinase (ZIPK) [34]. Additionally, both SAFB1 and SAFB2 genes (encoding scaffold attachment factor binding proteins that are localized in the nuclear matrix of the cell) are located at chromosome 19p13.3. Oesterreich et al. [35] found a markedly high fraction of LOH in 78% of informative DNA samples. Deletions in the same 19p region have also been reported by Lindblom et al. [36] in 27% of the studied tumors. The most common locus of LOH in the pancreatic intraductal papillary mucinous neoplasms (IPMNs) studied by Abe et al. [37] was at 19p. These neoplasms are closely associated with pancreatic adenocarcinomas.

On the long arm of chromosome 19 we observed losses in three cases including the well-known glioma tumor suppressor candidate region. This locus, which is frequently lost in glioma [38], includes the genes GLTSCR1 and GLTSCR2/PICT-1. Alterations in GLTSCR1 were associated with the development and progression of oligodendroglioma [39] and a polymorphism within the gene has been associated with prostate carcinoma aggressiveness [40]. Glioma tumour-suppressor candidate region gene 2 (*GLTSCR2*/*PICT-1*) is found to be down-regulated in glioblastomas and is sequentially lowered according to the histological malignant progression of astrocytic glial tumor [41]. GLTSCR2 induces PTEN-dependent apoptotic cell death and its inactivation might result in increased tumor growth due to decreased apoptosis in glioblastoma cells [42].

Cadherin-11 and fascin have been reported to be increased in the tumor front of primary PAs of patients with later recurrence [43]. The fascin gene is located at 7p22. It is a 55kDa actin-bundling protein and has received considerable attention as a prognostic marker of metastatic disease [44]. In one case we observed a gain of genetic material at this locus, supporting the assumed involvement of fascin in PA-development.

Microscopically, pleomorphic adenomas show a marked histological diversity with epithelial, myoepithelial and mesenchymal components in a variety of patterns. It would be reasonable to assume that the observed histological diversity may be due to a similar genetic diversity, as the one attested by the present study.

It should be noted that all of the aforementioned tumor types in which losses at 19p or 19p13 were discovered involve glandular epithelium. Based on these findings, one could

initiate the hypothesis that losses at the 19p (or even, more specifically, at the 19p13) may be associated with the development of carcinomas stemming from glandular epithelium. This hypothesis needs further testing. In addition, the 19p arm and especially its 19p13 region deserve further study by those interested in the genetics of pleomorphic adenomas.

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