Molecular cytogenetic pilot study on pleomorphic adenomas of salivary glands

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Abstract. Pleomorphic adenomas (PAs) of salivary glands are the most frequent entity of solid parotid tumors. Nonetheless, their genetics is not yet well understood. Thus, the current study characterized 14 PAs using a unique combination of cytogenetic, molecular cytogenetic and/or molecular karyotyping based approaches. The current study applied G-banding based on trypsin treatment and Giemsa-staining in peripheral blood and tumor tissue. Additionally, fluorescence *in situ* hybridization was performed using whole chromosome painting or centromeric probes. Array-based comparative genomic hybridization was also conducted. In 5 of 14 cases, chromosomal and/or submicroscopic alterations were characterized. Balanced and unbalanced translocations, loss or gain of whole chromosomes and submicroscopic copy number alterations were detected. Furthermore, the first case of a so-called 'jumping translocation' in a PA was reported. The genes twist-related protein 1 and distal-less homeobox 5 were also involved in copy number variations in two PAs. In conclusion, approaches utilized in the current study are highly suited to characterize the genetic constitution of PAs.

Introduction

Pleomorphic adenomas (PAs) account for 45.5% of primary salivary gland tumors. At the same time, 60-70% of parotid tumors are Pas (1). European-wide annual incidence of PAs is 4.2-4.9 per 100,000 inhabitants and year (2). PAs are slowly growing tumors which may remain asymptomatic and unrecognized over years, but they also can reach gigantic sizes and, if left untreated, are going together with dysphagia, dyspnea, great morbidity, or even malignant transformation (2,3). After parotidectomy, 2-3% of cases show local recurrences, while recurrence rates of up to 25-45% occurred after tumor enucleation (4). Accordingly, few remaining PA-derived tumor cells obviously are sufficient to form recurrent tumor nodules. Since even small resection margins increase the recurrence risk, the standard procedure for PA resection is meanwhile parotidectomy (5,6).

In addition to the influence of surgical technique on recurrence rates, the subtype of the PAs has an influence on prognosis; e.g. myxoid subtype PAs are more adverse than others (7). Other histopathological features, such as a thin tumor capsule, an incomplete capsule surface, pseudopodia (tumor nodules within the tumor capsule separated by a fibrous layer from the actual tumor) or satellite nodules (tumor nodules separated by healthy glandular tissue or fat from the tumor) also predispose to recurrences after PA resection. Additionally, size of the tumor influences later recurrences: Tumors being initially larger have higher recurrence rates than smaller ones (7-9). Finally, young age at first appearance and female gender also predispose to PA recurrences. As all previously mentioned data is descriptive and provide only statistical data, it is still unclear which genetic or molecular-biological causes are responsible for individual recurrence rates and which pathomechanisms enable individual cells of the PAs to develop recurrences.

Immunohistochemical or molecular genetic studies on expression of various receptors and proteins involved in signal transduction pathways were already performed in PAs. The role of Ki67 as a proliferation marker in recurrences remained hereby unclear (10), while an increased expression of some mucin glycoproteins appeared to be prognostic factors (11). Progesterone receptor but not estrogen receptor expression,

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was increased in recurrent PAs compared to the primary tumors (10). Also discussed as cause of recurrences in PAs is the density and incidence of lymphoid and blood vessels in the tumor itself and its environment (12). More recently, *PLAG1* and *HMGA2* fusion genes have been the focus of research in PAs. Both in PAs and in their recurrences enhanced *PLAG1* expression could be detected (13).

Surprisingly, basic cytogenetic and molecular cytogenetic studies in PAs are also scarce. Majority of cytogenetic studies showed normal karyotypes in a certain subset of the tested tumors (14-16); a more recent review even claims 30% of PAs show a normal karyotype (17). However, involvement of chromosomal breakpoints in 8q12, 6p21 and 12q15, have been reported, too (18). According to (19) no cytogenetic differences could be found in PAs deriving from minor versus such derived from major salivary glands. Molecular cytogenetics based on fluorescence in situ hybridization (FISH) (20,21) is not that frequently applied for research purposes in PAs as well, while some specific FISH-probes for above mentioned loci are routinely used (22). To the best of our knowledge no molecular karyotyping (array-comparative genomic hybridization = aCGH) studies have yet been undertaken for PAs.

Overall, there is still a lack of evidence for causal events of recurrence in PAs. Thus, in this pilot study we used for the first time a combination of cytogenetics, FISH and aCGH to characterize genetic alterations being present in overall 14 PAs.

Materials and methods

Material. Peripheral blood and primary tumor material were taken with written informed consent from 14 patients with PA of salivary gland beween October 2017 and December 2018 (Table I); only cases were included which were classified and confirmed by histopathology and immunohistochemistry as PAs (data not shown). Cells were either subjected to tissue culture following standard procedures, or frozen at -20˚C.

Cell culture and cytogenetics. Cells from tumor tissue were disassociated by collagenase treatment. The resulting cell suspension was transferred into in situ culture for 2-3 weeks. After trypsin treatment to remove adherent cells from tissue flasks, chromosomes were prepared as previously reported (23). Peripheral blood was cultured for 72 h and prepared as well as described before (23). In both cases the resulting so-called 'suspension' (methanol/acetic acid 3:1) was dropped on slides, thus spreading the obtained metaphases using the 'air-drying method' (23,24). G-banding based on trypsin treatment and Giemsa-staining (GTG) was applied to achieve banded chromosomes from primary tumor material as well as peripheral blood lymphocytes of each of the 14 patients. Ten metaphases were analyzed per case and tissue. Karyotypes were described according to ISCN 2016 (25).

Molecular cytogenetics. Fluorescence in situ hybridization (FISH) using all 24 human whole chromosome painting probes in one experiment [homemade M-FISH probe set (26)] was applied in cytogenetic preparations from tumor material of cases 2 and 3. Also, centromeric probe D17Z1 (Abbott/Vysis, Wiesbaden, Germany) was used in case 4. The FISH-procedures was done as reported in (27). Ten metaphases were analyzed after M-FISH and 20 metaphases after application of D17Z1.

Molecular karyotyping. Whole genomic DNA was extracted from tumor and blood using commercially available kits. This DNA was applied in two selected cases for array-based comparative genomic hybridization (aCGH, Agilent Human Genome CGH Microarray 180K); the blood-derived DNA was used as individual, case specific control for the tumor-derived DNA. aCGH was done as previously described (28).

Results

In all 14 studied PA-patients no constitutional chromosomal aberrations were detected after GTG-banding of peripheral blood derived T-lymphocytes. Also in 10/14 PA-derived tumor cells normal karyotypes were observed (Table I).

Aberrations were found as follows using a combination of GTG-banding, molecular cytogenetics and/or aCGH (see also Table I):

Case 1. Banding cytogenetics revealed an apparently balanced reciprocal translocation between chromosomes 11p11.2 and 12q14.3; according to aCGH the break-events in chromosomes 11 and 12 additionally involved a 107.46 and an 809.68kb deletion, respectively.

Case 2. Here GTG-banding and M-FISH identified two potentially related clones being present in this tumor with two different balanced translocations: in common was a breakpoint in 8q21.1. In 6/20 cells the latter region was fused to 3q29 and in the remainder 14 cells 8q21.1 was fused to 6q27. As both locations were (sub-) telomeric this may be considered as a so-called jumping translocation.

Case 3. The complex aberrations being present here only could be resolved after M-FISH (Fig. 1). Besides trisomy 7, also a balanced translocation between an X-chromosome and chromosome 8 and two additional marker chromosomes were observed. The latter turned out to be identical products of an unbalanced translocation between chromosomes 1 and 5.

Case 4. Banding cytogenetics detected 3/10 cells with monosomy 17; this finding could be confirmed using a centromeric probe for chromosome 17 and evaluating 20 more metaphases-overall loss of one chromosome 17 was present in 23% of the tumor cells.

Cases 1 and 12, assessed via molecular karyotyping. In this pilot study, only two cases were studied by aCGH. One with a cytogenetic detectable aberration (case 1) and one with a normal GTG-banding karyotype (case 12). In case 1 a ~0.11 and ~0.81 Mb deletion in the breakpoint regions 11p11.2 and 12q14.3 were found including tumor suppressor genes *WIF1* and *MEG3*. In case 12 a 0.5 Mb deletion was observed in 16p13.3 comprising among others also the tumor suppressor gene *STUB1*. Besides, in case 1 seven copy number variations (CNVs) between 0.23 and 22.27 kb in size were detected - all apart from that in 7p21.1 were heterozygote losses; and in case 2 seventeen CNVs (four losses and thirteen gains) between 0.07 and 42.13 kb were seen. In both cases DNA extracted from peripheral blood of the corresponding cases were used as controls in aCGH. Thus, even small CNVs should be meaningful (Tables II and III). According to Table III, in

Table I. The age, sex and (molecular) cytogenetic and aCGH results are listed together with other diagnoses (if available) of the corresponding 14 patients with pleomorphic adenoma.

Case number	Sex	Age (years)	*De novo*/ recurrence	(Molecular) Cytogenetics	aCGH	Other diagnoses
1	F	37	*De novo*	46,XX,t(11;12)(p12;q14.3)[10]	chr11:37.785.083-37.892.542 chr12:65,331,276-66,140,959xsee also Tab. 2	None
2	F	61	*De novo*	46,XX,t(3;8)(q29;q21.1)[6]/46,XX,t(6;8)(q27;q21.1)[14]	n.d.	Raynaud-syndrome, SHARP-syndrome
3	F	59	*De novo*	49,X,t(X;8)(p11.21;q12),+der(5)t(1;5)(q12;q11.2)x2,+7[cp20]	n.d.	Multiple Sclerosis
4	F	64	*De novo*	45,XX,-17[7]/46,XY[24]	n.d.	Von Willebrand-syndrome
5	F	54	*De novo*	46,XX[cp10]	n.d.	None
6	M	55	1. Recurr.	46,XY[cp10]	n.d.	None
7	M	54	6. Recurr.	46,XY[cp10]	n.d.	None
8	F	50	*De novo*	46,XX[cp10]	n.d.	None
9	M	55	2. Recurr.	46,XY[cp10]	n.d.	None
10	F	52	*De novo*	46,XX[cp10]	n.d.	None
11	M	41	4. Recurr.	46,XY[cp10]	n.d.	None
12	F	69	1. Recurr.	46,XX[10]	See Table II	None
13	F	65	3. Recurr.	46,XX[10]	n.d.	None
14	M	48	*De novo*	46,XY[10]	n.d.	None

Abbreviations in karyotype formulas are written according to ISCN 2016 [23]. n.d., not determined; recurr., recurrence (number in front of 'recurr.' represents the number of recurrences); F, female; M, male; aCGH, array-based comparative genomic hybridization.

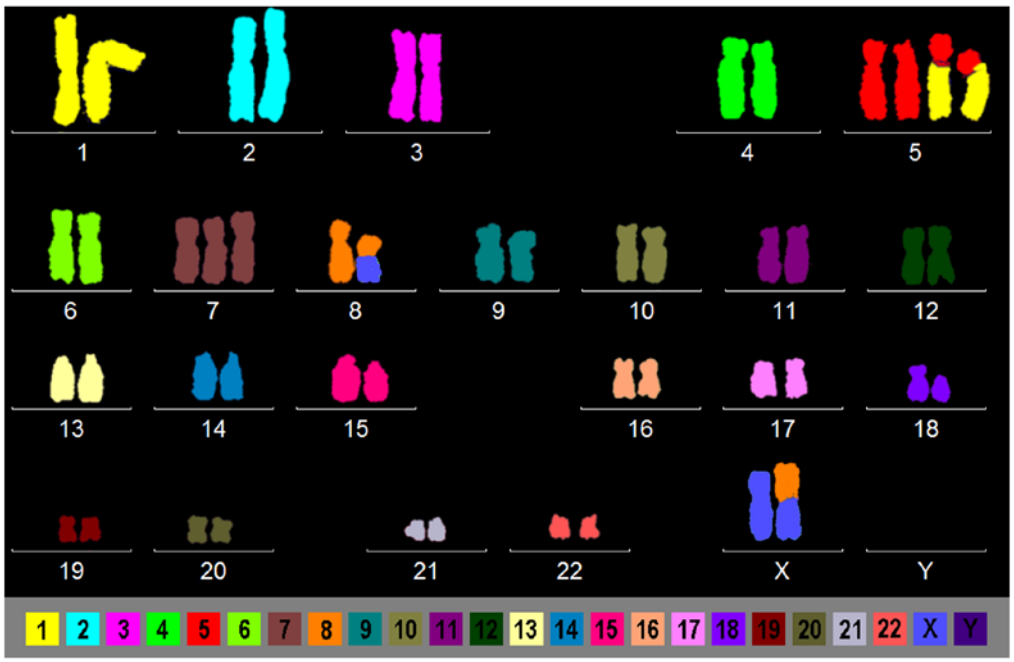

Figure 1. Multiplex fluorescence *in situ* hybridization results for pleomorphic adenoma tumor cells in case 3. Two additional derivatives of chromosome 5 (+der(5)t(1;5)(q12;q11.2)x2), one additional copy of chromosome 7 (+7) and a reciprocal translocation between the X-chromosome and one chromosome 8 were observed as acquired aberrations.

Table II. Results of array-based comparative genomic hybridization.

Case	Chr	Loss [GRCh37]	Gain [GRCh37]	CNV size [kb]
1	2	2p23.1(31,806,230-31,807,281)	7p21.1(19,155,127-19,155,358)	1.05
	7	7q21.3(96,651,603-96,655,351)		0.23
	11	11p15.5(2,015,691-2,020,975)		3.75
	12	11p12(37,785,083-37,892,542)		5.28
	14	12q14.3(65,331,276-66,140,959)		107.46
	17	14q32.2(101,290,932-101,295,092)		809.68
	20	17q24.3(70,118,098-70,120,417)		4.16
		20q11.22(34,006,276-34,028,549)		2.32
				22.27
12	1		1p36.13(16,345,776-16,387,906)	1.33
			1p31.3(68,516,381-68,517,713)	0.23
	2	2q31.1(172,964,377-172,964,608)		0.21
	7		7p21.1(19,156,027-19,156,233)	8.51
			7q11.23(73,485,261-73,493,768)	2.93
			7q21.3(96,652,421-96,655,351)	33.52
			7q34(142,453,637-142,487,154)	11.76
	8		8p11.1(43,371,449-43,383,206)	18.54
			8q11.1(46,939,154-47,457,692)	1.97
	11			0.32
	15	11p15.4(2,904,944-2,906,912)		0.07
		15q11.2(23,930,537-23,930,860)	15q15.3(43,850,909-43,850,979)	526.88
	16			2.51
	19	16p13.3(433,219-960,098)	19q13.43(57,348,729-57,351,242)	22.02
	20		20q11.22(34,006,276-34,028,297)	1.00
			20q11.23(36,150,802-36,151,799)	0.33
		20q13.32(57,464,121-57,465,999)	20q13.12(42,184,995-42,185,326)	1.878

Gains and losses were detected in cases 1 and 12 when comparing tumors with blood derived DNA. Chr, chromosome; CNV, copy number variation.

case 1 six out of nine CNVs cover a cancer related gene; in case 12 the rate is about the same: here 15/18 CNVs were correlated with tumor related genes in literature. Interestingly in both cases a gain of copy numbers involved the *TWIST* gene in chromosome 7p21.1 as well gain or loss, respectively for gene *DLX5* in 7q21.3.

Discussion

Genetic studies on PAs of salivary gland are scarce. To provide closing this gap, here we provided a yet unique cytogenetic, molecular cytogenetic and molecular karyotyping (aCGH) based pilot approach in 14 PA cases, to learn more about underlying acquired genetic changes in this cancer entity.

First we could confirm that a substantial part of PAs does not harbor any cytogenetically visible alterations. In contrast to the literature we found in GTG-banding normal karyotypes in 4/14 (~70%) of our cases and not in only 30% as previously suggested by others (17). However, this may have to be attributed to small sample size. Also we could, due to financial issues, in this pilot study only test two selected cases by aCGH. More cryptic unbalanced aberrations may also be present in the other 12 cases.

Additionally we could find several, completely different aberrations in the 4 cases, where cytogenetically visible aberrations were substantiated. As in previous reports, cytogenetically balanced and unbalanced chromosomal rearrangements, as well as numerical aberrations as gains or losses were present (Table I) (14-22). E.g. the loss of chromosome 17 going together with loss of one copy of tumor suppressor gene *TP53* has previously been seen in Pas (17).

Besides, this study showed the strengths of the chosen approach, i.e. combining banding cytogenetics with FISH and/or aCGH. Thus, it was easily possible either to better characterize and/or resolve karyotypic changes not to be clarified by GTG-banding alone, like in cases 2 and 3. Similar observations have been made previously for other tumors (29), but not or only rarely for Pas (20). Interestingly in case 2 one of the rare instances of a so-called jumping translocation could be observed; mechanism and meaning for pathology are unclear, still it is the first such observation in a PA (30).

Table III. Genes involved in the detected CNVs of cases 1 and 12.

Case	CNVs [GRCh37]	Tumor related genes
1	2p23.1(31,806,230-31,807,281)	n.a.
	7p21.1(19,155,127-19,155,358)	TSG: *TWIST1*
	7q21.3(96,651,603-96,655,351)	?TSG: *DLX5*
	11p15.5(2,015,691-2,020,975)	?OG: *H19*
	11p12(37,785,083-37,892,542)	n.a.
	12q14.3(65,331,276-66,140,959)	TSG: *WIF1*
	14q32.2(101,290,932-101,295,092)	TSG: *MEG3*
	17q24.3(70,118,098-70,120,417)	n.a.
	20q11.22(34,006,276-34,028,549)	?OG: *GDF5*
12	***1p36.13(16,345,776-16,387,906)***	?TSG: *HSPB7*
	1p31.3(68,516,381-68,517,713)	?TSG: *DIRAS3*
	2q31.1(172,964,377-172,964,608)	?TSG: *DLX2*
	7p21.1(19,156,027-19,156,233)	TSG: *TWIST1*
	7q11.23(73,485,261-73,493,768)	n.a.
	7q21.3(96,652,421-96,655,351)	?TSG: *DLX5*
	7q34(142,453,637-142,487,154)	?TSG/OG:*TCRVB*
	8p11.1(43,371,449-43,383,206)	n.a.
	8q11.1(46,939,154-47,457,692)	n.a.
	11p15.4(2,904,944-2,906,912)	TSG: *CDKN1C*
	15q11.2(23,930,537-23,930,860)	?TSG: *NDN*
	15q15.3(43,850,909-43,850,979)	?TSG: *PPIP5K1*
	16p13.3(433,219-960,098)	?TSG: *PPIP5K1*
		?TSG/OG: *RAB11FIP3*
		?TSG/OG: *RAB40C*
		TSG: *STUB1*
		?TSG/OG: *JMJD8*
		?TSG/OG: *METRN*
		?TSG/OG: *MSLN*
	19q13.43(57,348,729-57,351,242)	?CIN: *CHTF18*
	20q11.22(34,006,276-34,028,297)	?TSG/OG: *PEG3*
	20q11.23(36,150,802-36,151,799)	?TSG/OG: *GDF5*
	20q13.12(42,184,995-42,185,326)	TSG: *BLCAP*
	20q13.32(57,464,121-57,465,999)	OG: *SGK2*
		?TSG/OG: *GNAS*

CNVs detected in cases 1 and 12 by aCGH and the gene located there, which were associated with tumors according to a combined search of UCSC (GRCh37) (genome-euro.ucsc.edu) and pubmed (https://www.ncbi.nlm.nih.gov/pubmed/). Regions present in three copies in the corresponding patient are highlighted in italics and bold letters. CNV, copy number variation; n.a., no tumor related or no gene listed in UCSC; OG, oncogene; TSG, tumor suppressor gene; ?, questionable; CIN, gene correlated with chromosome instability.

Also, cryptic submicroscopic changes could be picked up by applying aCGH in cases 1 and 12. As listed in Table III, in 6/9 to 15/18 of the detected CNVs according to the literature tumor related genes were located. Also *TWIST1* and *DLX5* genes were involved in CNVs in both by aCGH studied PAs. Thus, here further studies towards the role of these genes in PAs may be indicated.

In conclusion, the setting of pilot study in 14 PAs showed that the combination of banding cytogenetics, FISH and aCGH, maybe in future enlarged by other approaches like MALDI-MS imaging (MSI) (31) enable completely new insights into the genetics of this yet understudied tumor entity. Cryptic and submicroscopic chromosomal aberrations can be picked up more reliably by such an approach.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JT and OGL provided patient material and clinical data. JT, OGL, FVE, AW and TL developed and planned the current study. AW performed cytogenetic analyses and interpretation. MAKO performed DNA-extraction and final aCGH evaluation. MZ conducted the molecular cytogenic experiments. JBM and IMC performed aCGH studies and first evaluation. All authors wrote and approved the final draft of the manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethical board of the Friedrich Schiller University, medical faculty (approval no. 5241-08/17). Patient consent for participation in the current study was obtained from each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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