Chromosomal gains and losses indicate oncogene and tumor suppressor gene candidates in salivary gland tumors

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The incidence of salivary gland tumor in Poland is growing in the last two decades. Simultaneously a progress in understanding the genetic mechanisms of formation of this tumor was achieved by detecting several genes like *PLAG1* involved in its pathogenesis. In this study we perform a whole genome, CGH analysis with the aim to identify recurrent, chromosomal copy number changes possibly indicating novel tumor suppressor gene or oncogene loci.

29 salivary tumor samples: Cystadenolymphoma-warthin (15) and *adenoma polymorphum* (14) located in the parotid (27) and submandibular gland (2) were collected and CGH was performed. The established copy number profiles were compared in order to asses the smallest common region of gains and losses. The delineated regions were further analyzed with the UCSC Genome Browser on Human Mar. 2006 Assembly to asses their gene content.

Altogether, salivary gland tumors presented a different aberration pattern than these reported for head and neck squamous cell carcinoma (HNSCC) but no significant differences were observed between *Warthin* and *adenoma polymorphum* tumors. Moreover, several potential tumor suppressor genes and oncogenes were identified in the smallest, common altered regions. We show a frequent deletion of the *harakiri* gene (12q24.2) in 12/29 tumors and *TP53* gene (17p13.1) in 11/29 tumors as potential tumor suppressors in salivary gland cancers. Besides, we detected a frequent amplification of the 13q22.1-22.2 region in 13/29 cases harboring the *KLF5* and *KLF12* genes. *KLF5* regulates the expression of *survivin*, an oncogene widely expressed in the majority of human cancers. The observed alterations may indicate important genetic events in the formation of salivary gland tumors. Especially the amplification in 13q may be a mechanism contributing to the expression of *survivin* and tumor progression.

Keywords: salivary gland tumors; chromosomal instability; TSG; CGH; KLF5; TP53

Pleomorphic adenoma and *cystadenolymphoma* (*Warthin*) are the most common benign neoplasms of the salivary gland with the typical localization in the parotid or submandibular gland [1, 2]. The incidence of salivary gland tumors in Poland is 0.8 for men and 0.7 for women per 100 000 persons [3]. In the years 1985-1995 in the Clinic of Otolaryngology and Laryngological Oncology at the K. Marcinkowski Medical University in Poznań 7–16 patients/year were treated on salivary gland tumors. In the years 2002-2004 already 70-74 patients/year were treated that indicates a 6 fold increase in the incidence rate observed in our clinic during a time span of 20 years. A study embracing northern Poland shows a rapid increase of benign parotid gland tumors incidence from 25-30 cases/year in the years 1991-1996 to 50-60 cases/year in 1998-2000 [4]. This observation is further supported by the general tendency noticed in Poland. The incidence of tumors of large salivary glands in 1994 was 0.3 for males and 0.1 for females per 100 000. However, the incidence in 2000 was already 0.7 for males and 0.4 for females per 100 000. Noteworthy, the overall cancer incidence in Poland dropped from 428.7 / 100 000 in 1994 to 416 / 100 000 in 2000 [5, 6]. Although the increase in salivary gland tumor incidence in Poland may be partially caused by the growing consciousness on cancer threat or on better diagnostic procedures other studies point towards full mouth dental X-rays, occupational exposure to radioactive material or nickel compounds as possible risk factors [7].

In the last two decades a progress in understanding genetic mechanisms of salivary gland tumor formation was achieved.

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Table 1. Characteristics of salivary gland tumor patients F-female; M-Male; L-left; R-right

n	name	age/sex	localization		tumor diameter at surgery	histiology
			parotid	submandubular		
1	**P1**	54/F	L		5cm	pleomorphic adenoma
2	**P2**	56/F		L	3cm	pleomorphic adenoma
3	**P3**	27/F	R		2.5cm	pleomorphic adenoma
4	**P4**	32/F		R	3cm	pleomorphic adenoma
5	**P19A**	70/F	L		3 cm	Wartihn
6	**P19B**	70/F	R		3 cm	Warthin
7	**P48A**	53/M	L		4 cm	Warthin
8	**P48B**	54/M	R		3 cm	Warthin
9	**P11A**	47/F	L		3cm	Warthin
10	**P11B**	47/F	R		3cm	Warthin
11	**P12**	54/F	R		6cm	pleomorphic adenoma
12	**P13**	67/F	R		3cm	Warthin
13	**P14**	?/M	L		2cm	Warthin
14	**P16**	?/M	R		4cm	pleomorphic adenoma
15	**P19**	61/M	L		2cm	Warthin
16	**P20**	41/M	L		1.6cm	Warthin
17	**P21**	64/M	L		4.5cm	Warthin
18	**P22**	75/F	R		2cm	pleomorphic adenoma
19	**P24**	67/M	L		2cm	pleomorphic adenoma
20	**P25**	75/F	R		3cm	Warthin
21	**P26**	63/M	L		3cm	Warthin
22	**P27**	54/F	L		25cm	pleomorphic adenoma
23	**P28**	51/F	L		4.5cm	pleomorphic adenoma
24	**P29**	50/M	L		2.5cm	pleomorphic adenoma
25	**P30**	70/M	R		2cm	Warthin
26	**P31**	48/M	L		4cm	pleomorphic adenoma
27	**P32**	59/F	R		5cm	pleomorphic adenoma
28	**P33**	57/M	R		2cm	Warthin
29	**P34**	53/F	L		4cm	pleomorphic adenoma

Several chromosomal regions have been reported to be recurrently altered in salivary gland tumors and candidate genes have been proposed. The tumor suppressor genes (TSG) *PLAG1* (8q12) and *PLAGL1* (6q23-25) were shown to be rearranged in *pleomorphic adenomas*. Moreover, it was suggested that *HMGIC* (12q15) a transcription regulation factor frequently deleted in lipomas may be involved in the pathogenesis of salivary gland tumors [8, 9, 10]. Kish et al. suggested promoter methylation as a putative deactivation mechanism of *RB1* (13q14) [11]. Besides, the significance of *TP53* alterations has been also shown [12].

In this study we present a whole genome analysis of 29 salivary gland tumors towards recurring chromosomal gains and losses using the CGH technique. A comparison of copy number changes between *Warthin* tumor and *pleomorphic adenoma* are presented. Finally, several recurrently altered regions were analyzed and target TSG and oncogene candidates considered.

Materials and Methods

29 fresh frozen (P1-P4, P12-P14, P16) or paraffin embedded (P19A, P19B, P48A, P48B, P11A, P11B, P19-P22, P24-P34) tumor samples were collected from the Clinic of Otolaryngology and Laryngological Oncology at the K. Marcinkowski Medical University in Poznań / Poland. The cohort included 14 *pleomorphic adenoma* tumors, 15 *Warthin* tumors (Tab. 1).

Standard phenol/chloroform DNA extraction method was used and CGH was performed as described elsewhere [13]. Briefly, tumor DNA and normal male or female reference DNA were labeled by nick translation with Fluorescein-12-dUTP (Roche Diagnostics GmbH Mannheim, Germany) or Biotin-16-dUTP (Roche Diagnostics GmbH Mannheim, Germany) and visualized by Fluorescein-Avidin D and Biotynylated-Anti-Avidin D antibodies (Vector laboratories Inc. Burlingamo, CA 94010) and Tetramethyl-rhodamine-5-dUTP (Roche Diagnostics GmbH Mannheim, Germany), respectively. A mixture of both DNAs and COT Human DNA (Roche Diagnostics GmbH Mannheim, Germany) was hybridized onto CGH Target Slides – Normal Methaphase (Vysis Inc. Dovners Grove IL 60515 USA) for 48 hours. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with Vectashield antifading buffer (Vector Laboratories, Burlingame, CA). Hybridized slides were analyzed using an Olympus fluores-

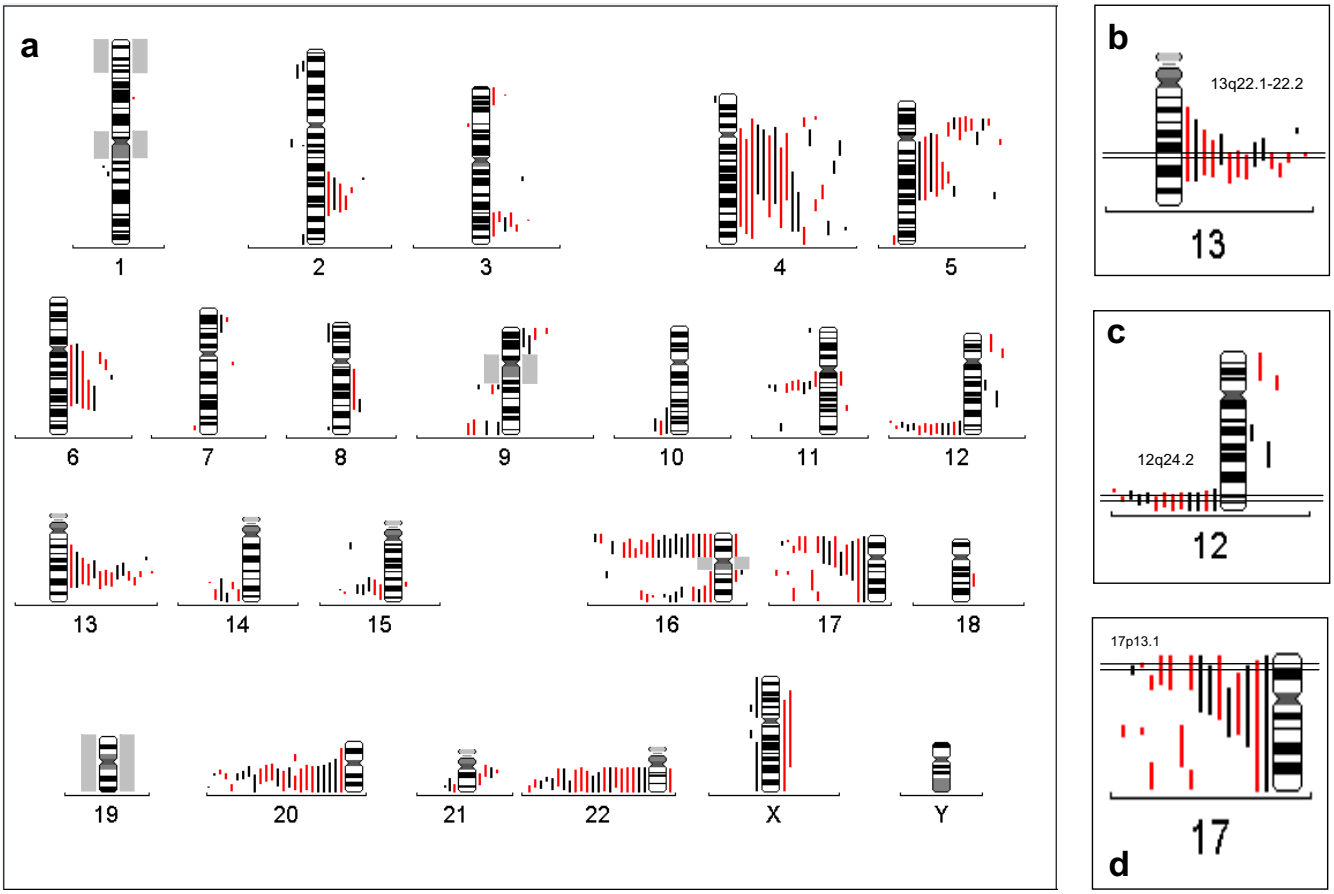

Fig. 1. Chromosomal aberrations found in 29 CGH analyzed salivary gland tumors
a. Bars on the left side of chromosomes represent losses; on the right side represent gains. Bars interrupted by regions indicating no aberrations were joined to the best of our knowledge. Aberration in *adenoma polymorphum* tumors are marked with black bars; aberration in *Warthin* tumors are marked with red bars; **b.** smallest, common, amplified region on chromosome 13; **c.** smallest, common, deleted region on chromosome 12; **d.** smallest, common, deleted region on chromosome 17.
Note: The X chromosome was analyzed only in 15 cases.

cence microscope assisted by a computerized ISIS digital image analysis system (MetaSystems Hard & Software, Altlussheim, Germany) and copy number profiles were obtained. The copy number profiles of analyzed cases were printed onto one ideogram and the smallest, common regions of gain or loss were assessed manually to the best of our knowledge. In this way identyfied recurrent regions of gains and losses were analyzed for the presence of potential tumor suppressor genes and oncogenes using the UCSC Genome Browser on Human Mar. 2006 Assembly.

Results

A nonrandom distribution of chromosomal aberrations among 29 analyzed tumor samples was found with several recurrent gains and losses (Tab. 1, Fig. 1). Most frequent losses were localized to 22q13 (21/29); 16q12 (17/29); 12q24.2 (12/29) and gains localized to 4q (19/29) and 13q22.1-22.2 (13/29). All found, recurrent chromosomal aberrations and genes potentially implicated in carcinogenesis located in these regions are presented in Table 2.

A comparison of the distribution of found copy number changes in the two histopathological groups – *pleomorphic adenoma* and *Warthin* tumor – was performed. No significant changes in frequency or in the distribution of chromosomal aberrations were identified (Fig. 1). The smallest, common aberrant region could be precisely delineated for the 11q13.3 and 12q24.2 deletions and 13q22.1-22.2 amplifications. Moreover, the frequency of *TP53* (17p13.1) deletion distribution in the studied samples was analyzed (Tab. 3).

In order to test the reliability of CGH we performed a normal female DNA to normal male DNA hybridization. In this experiment we identified the following aberrations: the expected gain of chromosome X as a positive control and besides, loss of 1p33-pter, loss of the centromeric re-

Table 2. Most frequent, recurrent chromosomal aberrations and putative target genes found in 29 CGH analyzed salivary gland tumors

Deletions			Amplifications		
Chr	**cases/29 (%)**	**Genes possibly implicated in cancer**	**Chr**	**cases/29 (%)**	**Genes possibly implicated in cancer**
11q13.3	8 (28%)	***CCNA1***, *LHFP*	**4q**	19 (66%)	*?*
12q24.2	12 (41%)	***Hrk***, *SUDS3*	**5p14.1**	7 (24%)	*CDH9*
15q25	6 (21%)	*?*	**5q**	8 (28%)	?
16p12	17 (59%)	*?*	**6q**	8 (28%)	?
16q24.1	11 (38%)	*?*	**13q22.1-22.2**	13 (45%)	***KLF5, KLF12***
17p13.1	11 (38%)	***TP53***			
22q13	21 (72%)	?			

Table 3. Distribution of aberrations in 17p13.1 in analyzed cases sorted according to the increase of tumor diameter.

Sample	del 17p13.1	Tumor diameter at surgery
P20	**Yes**	**1,6**
P14	No	2
P19	No	2
P22	**Yes**	**2**
P24	No	2
P30	**Yes**	**2**
P33	No	2
P3	No	2,5
P29	No	2,5
P2	No	3
P4	No	3
P11A	No	3
P11B	No	3
P13	No	3
P19A	No	3
P19B	No	3
P25	**Yes**	**3**
P26	No	3
P48B	No	3
P16	**Yes**	**4**
P31	**Yes**	**4**
P34	No	4
P48A	**Yes**	**4**
P21	**Yes**	**4,5**
P28	No	4,5
P1	**Yes**	**5**
P32	No	5
P12	No	6
P27	**Yes**	**25**

gion of chromosome 1 and 16, gain of the centromeric region of chromosome 9, loss of 19p13.1-q13.3. All these regions were not scored in the results for reasons specified elsewhere [14]. Surprisingly, we identified also a loss in 20q11.2-q12 thus this region was excluded from further analysis.

Discussion

In the cohort of 29 analyzed samples a repetitive pattern of chromosomal gains and losses was found which was different than that described for head and neck squamous cell carcinoma [15, 16]. Thus, salivary gland tumors should be treated as a distinctive entity in head and neck tumors. Within the cohort of analyzed salivary gland tumors no significant differences were found between *Warthin* tumors (15) and *pleomorphic adenoma* tumors (14) suggesting similar aberrations patterns in both (Fig. 1).

Unfortunately, not all repetitive aberrations allowed a precise narrowing of the target chromosomal band. Repetitive amplification were identified in chromosomes 4, 5 and 6 and deletions in 16, and 22 but the smallest common region could not be identified in these instances. Some of these including 6q were reported previously by other groups as frequently altered in salivary gland tumors [17].

The smallest, common regions of gains and losses were analyzed for putative genes involved in tumor pathogenesis (Tab. 2). Among these a deletion of 11q13.3 was observed. Interestingly, an amplification of this locus is a hallmark of HNSCC [18, 19]. The deletion of this region stresses the differences between the diverse groups of head and neck cancers.

A precise delineation of the smallest amplified region was possible for chromosome 13. The amp13q22.1-22.2 was identified in 45% of analyzed cases indicating the potential importance of this aberration in the formation of salivary gland cancers. This regions harbors among others the *KLF5* and *KLF12* transcription factors as putative target genes of the amplification. It has been shown recently that *KLF5* a gene widely expressed in acute lymphoblastic leukemia (ALL), interacts with *TP53* gene and regulates the expression of *survivin.* Furthermore, *survivin* that is reported to be expressed in almost all human cancers, inhibits apoptosis and plays a function in regulation of mitosis [20, 21]. Miyachi *et al*. showed that the expression of *survivin* is increased in gastric cancer tissues especially in patients displaying lymph node metastasis [22]. Recently, Qi *et al*. found a significant difference of *survivin* expression between benign and malignant tumors [23]. Summing up, *survivin* may be an important oncogene playing a role especially in late events of carcinogenesis like benign – malignant transition or metastasing and the 13q22.1-22.2 gain may be a late cytogenetics event. The identification of these aberrations in benign tumors in this study may be an important prognostic

marker but observations on patients survival is necessary to clear this. Finally, the recurrent amplification of the *KLF5* gene found in this study may present a mechanism explaining the up-regulation of *survivin* in salivary gland tumors.

Besides, we delineated a deletion in 12q24.2 harboring the harakiri (*Hrk*) apoptosis activator gene as potential deletion target and potential TSG in salivary gland tumors [24].

In the CGH profiles a recurrent deletion of the 17p13.1 region harboring the *TP53* gene was found. Interestingly, we observed that 17p13.1 loss was predominant in tumors of bigger diameter measured at the time of surgery. Loss was identified in 6/10 tumors of diameter ≥4cm but only in 4/19 tumors <4cm of diameter (Tab. 3). Our data harmonize with the findings of Yamamoto et al. who showed a *TP53* LOH in 57% of pleomorphic adenomas and 86% of carcinomas of pleomorphic adenomas [12]. These results show the relevance of *TP53* losses in the formation and progression of salivary gland tumors.

The increase of salivary gland tumors incidence in Poland creates a need for extensive studies to understand the genetic background of this disease. This study provides a group of candidate genes for TSG and oncogenes in salivary gland tumors. We suggest the importance of the *KLF5* gene in the maintenance of *survivin* oncogene expression. Besides, two TSGs were delineated, the *Hrk* gene of potential involvement in salivary gland tumors and the *TP53* gene that was found to be frequently deleted especially in tumors of bigger diameter. These findings, may possibly aid the diagnosis, prognosis and potentially therapy of patients with salivary gland tumors.

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