Genomic alterations in Warthin tumors of the parotid gland

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Abstract. Despite the fact that Warthin tumors are the second most common type of benign salivary gland tumors, information regarding genetic alterations is extremely limited, and the tumorigenesis of these tumors has not been elucidated. The present results of the largest series of 30 tumors analyzed by comparative genomic hybridization (CGH) to date confirmed previous genetic findings and identified significant new candidate regions. The most commonly observed alterations were deletions of the short arm of chromosome 8, followed by deletions on 9p. Further representative changes were deletions on 16p and 22q with the minimal overlapping region at 16p12p13.1 and 22q12.1q12.3. Moreover, we indicated two different patterns of chromosomal aberrations. One group harbors deletions on 8p partly apparent with deletions on 9q, 11q 15q, 16p and 22. The second group shows gains on 22, partly apparent with gains on 1p and 20q and deletions on 9p. This leads to the assumption that Warthin tumors, in particular those with a high number of alterations, can be divided into two different genetic groups based on the pattern of numerical chromosomal aberrations. Further studies should address whether these subgroups also reflect a different clinical presentation.

Introduction

Warthin tumors (WTs) are the second most common benign salivary gland tumors and account for ~15% of all epithelial parotid gland tumors. They are well encapsulated lesions with cystic and solid areas consisting of an oncocytic epithelial cell and a variable stroma component with lymphoid tissue. Malignant alteration is very rare and surgical resection is the most common treatment modality (1-3).

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To date, tumorigenesis of WTs is unknown. Two different theories for WT development are currently being discussed. One is the hypothesis of heterotopia, which implies that the tumor results from proliferating ductal cells of the salivary gland that were entrapped in parotid lymph nodes during embryonal life. The second theory suggests that a WT initially develops in the parotid lymph nodes as an adenomatous epithelial proliferation in response to a yet unidentified stimulus, such as tobacco, with concomitant lymphocytic infiltration (1). The putative link between tobacco consumption as one of the aetiologic factors associated with the development of WTs is in line with the observation that the incidence of WTs in smokers is eight times higher than that in non-smokers (4).

To date, only limited cytogenetic information is available for WTs. The majority of tumors analyzed to date by conventional cytogenetics have an apparently normal karyotype. Only 10% of WTs exhibit genetic alterations (5-13). Based on the detected genetic alterations, three main stemline groups are proposed for WTs: i) normal karyotype, ii) numerical changes only, i.e. loss of Y chromosome or trisomy/ monosomy 5, and iii) reciprocal translocations such as the t(11;19) translocation resulting in the *MAML2/CRTC1* fusion gene (11). This translocation is characteristic for mucoepidermoid carcinoma (MEC) while a possible derivation of certain MECs from WTs is under discussion (11-15). Identification of 6p translocations suggests that the short arm of chromosome 6 contains a region involved in the origin of WTs (6,8). Identification of clonal alterations in almost half of the cases further supports that this lesion is a 'true' neoplasm rather than an autoimmune or hypersensitivity related tumor-like proliferation, as previously suggested (16).

To the best of our knowledge, only one molecular cytogenetic study using comparative genomic hybridization (CGH) analysis on WTs has been published to date. The authors of this previous study observed several chromosomal gains and losses in a cohort of 15 tumors. Frequent chromosomal losses were reported at 12q (47%), 17p (53%) and 22q (73%), whereas frequent chromosomal gains were located at 4q (60%), 6q (33%) and 13q (67%) (17). This high frequency of detected alterations by molecular cytogenetic analysis compared to conventional cytogenetics underlines the need and importance for investigating native tumor material other than cell culture preparations to detect chromosomal alterations.

Herein, we present a second molecular cytogenetic study of WTs with the largest cohort of 30 tumors to date in order
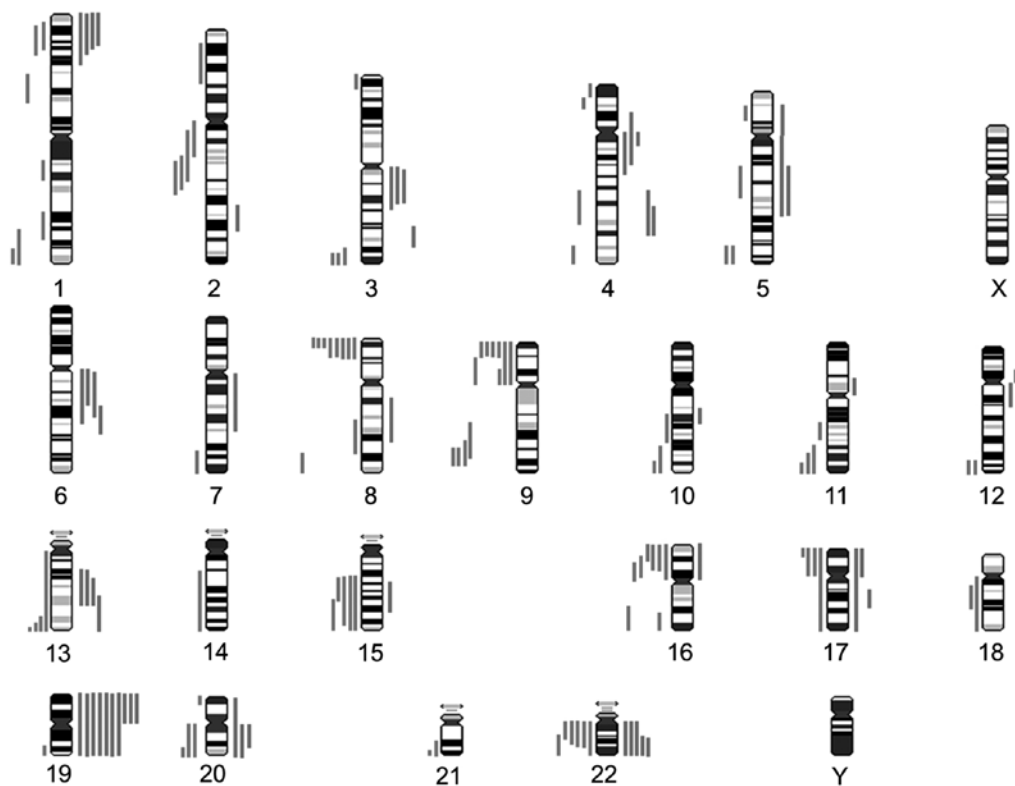
Figure 1. Summary of the chromosomal alterations identified in the Warthin tumors by CGH. Bars on the left side indicate losses of chromosomal material and bars on the right side represent gains.

to confirm previous reported alterations and to detect new significant chromosomal aberrations because of the higher number of tumor samples.

Materials and methods

Patient characteristics. A total of 30 patients (14 female and 16 male), with a median age of 57 years (19-87 years), who received a parotidectomy with the purpose to remove a parotid gland mass between the period 2000 and 2006 at the Department of Otolaryngology, University Hospital Homburg/ Saar, Germany was investigated. All surgeries were primary surgeries treating no recurrent disease. Prior to surgery, all patients were informed in regards to the protocol and provided their written consent for donating tissue for research purposes. Tissue was snap frozen immediately after removal at -80˚C for CGH analysis. The diagnosis of benign WT was confirmed by detailed histopathological evaluation in all tumors. To date, none of the patients has developed a tumor recurrence.

CGH. We obtained DNA using phenol/chloroform extraction. Tumor DNA and reference DNA were labeled with biotin and digoxigenin by nick translation according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Tumor DNA and reference DNA, 600 ng each, were hybridized with Cot1-DNA (Roche Diagnostics) to normal chromosome meta-phase spreads from peripheral blood lymphocytes prepared following standard procedures. After three days of hybridiza-tion at 37˚C, post-hybridization washes were performed at a stringency of 50% formamide/2X standard saline citrate

(SSC), 2X SSC and 0.1X SSC at 45˚C. Tumor DNA was visu-alized with fluorescein-isothiocyanate (Vector Laboratories, Inc., Burlingame, CA, USA), reference DNA with rhodamine (Roche Diagnostics) and counterstained with an anti-fade solution containing DAPI (4,6-diamidino-2-phenylindole) (Vector Laboratories). Fluorescence images were captured using an Olympus BX 61 fluorescence microscope with a cooled charged-coupled device camera. Image processing was performed by using the computerized ISIS digital image anal-ysis software system (MetaSystems, Altlussheim, Germany). Average ratio profiles were determined from analysis of 12-15 metaphases. The thresholds used for ratio profiles were 1.2 for gains, and 0.8 for losses.

Due to the suppression with Cot1-DNA, the fluorescence intensities were not representative at chromosome regions with tandem repetitive DNA clusters, that is at the heterochromatic blocks on chromosomes 1, 9, 16, and Y at the centromeric regions and along the short arms of acrocentric chromosomes. These areas and the whole sex chromosomes were excluded from CGH evaluation. Additionally, the chromosomal regions, such as 1p34pter, 19 and 22 were interpreted cautiously, particularly as they are prone to problems associated with the different hybridizability of their GC-rich regions (18).

Results

CGH. Chromosomal alterations were detected in a non-random distribution in 27 out of the 30 hybridized WTs by CGH analysis. The mean number of imbalances was 5.3 (range from 0 to 21). Detection of losses was more frequent

Table I. Results obtained from CGH analysis of 30 Warthin tumors.

Tumor	Age/ Gender	Chromosomal gains	Chromosomal losses	No. of gains	No. of losses	Total no. of imbalances
1	75/F	3q11q21, 4q28q31, 6q11q22.2, 12q11q13	22q11q12	4	1	5
2	56/F	3q11q13.3, 4q26q31.3, 8q13q22, 13q14q22		4	0	4
3	52F	-	-	0	0	0
4	58/M	5q14q23, 6q21q22	-	2	0	2
5	50/M	13q22qter		1	0	1
6	53/M	-	-	0	0	0
7	50/M	-	8q24.1qter	0	1	1
8	57/M	-	5p14	0	1	1
9	87/F	4q11q13.1,12q11q12	1p33p36.1, 16p11.1p12	2	2	4
19	56/M	19	-	2	0	2
20	39/M	19p	2q12q23, 8p23.1pter	1	2	3
25	56/M	-	9p11p21	0	1	1
26	51/M	-	1p22.1p31.1, 10q24.3qter	0	2	2
27	42/M	-	-	0	0	0
28	64/F	-	17p	0	1	1
22	71/F	<u>1p33pter</u>, 17p11pter, 19, <u>20q11.1q13.1</u>, <u>22q</u>	<u>9p22pter</u>, 13q33qter, 20p13pter	6	3	9
23	56/F	<u>1p32.3pter</u>, 19p, <u>22q12.2qter</u>	3q28qter, 4q33qter, 5q14q21, <u>9p11p13</u>, 9p23pter	3	5	8
24	66/F	1p34.1pter, 19, 20	1q43qter, 9p	5	2	7
29	19/M	<u>1p35pter</u>, 17p, 19, <u>22</u>	5p11p12	5	1	6
30	57/F	11p11.1p12, 19, <u>22</u>	2q21.3q24.3, 4q26q31.1	4	2	6
21	61/F	15q21.2q24, 17, 19, <u>20q</u>, <u>22q12.3qter</u>	2q21.1q24.2, 8p23.1pter, <u>9p</u>	6	3	9
10	61/F	2q32.1q33, 3q25.3q26.3, 4p11p14, 4q11q21.3	**15q21.1q22.3**, **16p13.1pter**, **22q11.1q13.1**	4	3	7
11	62/M	5p11p14, 5q11q23.3, 6q11q22.1	3p24.3pter, 5q34qter, 7q33qter, **8p23.1pter**, 9q32q34.1, **11q23.1qter**, 12q24.2qter, 13q32qter, **15q21.1qter**, **16p12pter**, 16q23qter, 17, 18q, 19q13.3qter, 20q, 21q22.1qter, **22q12.2qter**	3	18	21
12	45/F	3q11.2q13.3, 4q11q22, 6q12q21, 13q14.3q22	**8p23.1pter**, 9q32q34.1, 10q26.1qter, **11q23.3qter**, 12q24.2qter, 14q21qter, **15q23qter**, 16q22qter, 17p, 20q13.2qter, **22q11.1q13.2**	4	11	15
13	64/M	19p	1p34.1p36.2, 1q21.2q23, 1q32.1q41, 2p16p23, **8p21.3pter**, 8q21.3q23, 9q22.1q33, 10q22.1q24.1, **11q14.1q22.1**, **15q21.1q25**, **16p11.2p13.1**, 18q12.1q21.3, 20q, **22**	1	13	14
18	63/M	12p11p12.1	2q11.1q14.3, 3q28qter, **8p21.3pter**, **15q21.3qter, 22q11.1q12.3**		5	6
14	61/F	7q11.1q31.2, 10q21.2q22.2, 13q14.1q22	1q41qter, **8p22pter**, 16p	3	3	6

Table I. Continued.

Tumor	Age/ Gender	Chromosomal gains	Chromosomal losses	No. of gains	No. of losses	Total no. of imbalances
15	80/F	17q21.2q22, 19	5q34qter, 9p23pter, 13q34qter	3	3	6
16	57/M	-	3q27qter, 4p16pter, **8p22pter**, 9q31q34.1, **16p12pter**, 17p13pter, 21q22.3qter	0	7	7
17	46/M	19	**8p23.1pter**, 9p23pter, **11q24qter**	2	3	5

F, female; M, male. Tumors with a higher number of CNAs (6 or more) show two different patterns of alterations: one is characterized by deletions affecting 8p, 9q, 11q 15q, 16p and 22 (bold type); in the second one gains of 22 were partly apparent with gains on 1p and 20q and losses of 9p (underlined).

Figure 2. Consensus regions at chromosome arm 6q, 8p, 9p, 9q, 13q and 16p. Bars on the left side indicate losses of chromosomal material and bars on the right side represent gains.

Table II. Chromosomal alterations and putative target genes observed by CGH.

Chromosome	No. of cases/30 (%)	Candidate genes
Deletions		
2q	4 (13)	?
8p23.1pter	9 (30)	*ANGPT2, MCPH1, PINXI*
9p	7 (23)	*CDKN2A, CDKN2B*
9q	4 (13)	*TSC1, GAS1*
11q24qter	3 (10)	*TBRG1, CHEK1*
15q	5 (17)	?
16p	6 (20)	*MAPK3, ERCC4, LITAF*
17p	4 (13)	*TP53*
22q12.1q12.3	6 (20)	*CHEK2, TIMP3*
Gains		
4q	5 (17)	*EGF, C-Kit*
6q21	4 (13)	*FYN, HDAC2*
13q22	4 (13)	*KLF12*
22q	5 (17)	*PDGFB*

than gains (93 vs. 67, mean 3.1 vs. 2.3). The graphical representation of the genetic alterations is shown in Fig. 1. The clinical data and chromosomal imbalances are summarized in Table I.

The most commonly observed alterations were deletions of the short arm of chromosome 8 (30%), followed by deletions of 9p (23%). Further representative changes were deletions on chromosome arm 16p and 22q with the minimal overlapping region at 16p12p13.1 and 22q12.1q12.3 in 20% of the investigated tumors (Fig. 2). Moreover, in 17% of the cases, gains at 4q, 15q and 22q were detected. At a lower frequency, deletions on 2q, 9q and 17p and gains on 6q and 13q were observed (13% each), with the minimal overlapping regions at 13q22 and 6q21q22.1. An overview of the most commonly observed alterations and putative candidate genes is shown in Table II.

Our data indicate that almost all tumors with a higher number of copy number alterations (6 or more) are able to be distinguished by two different patterns of alterations. The first one is characterized by deletions affecting 8p, 9q, 11q 15q, 16p and 22, whereas in the second one gains of 22 were partly apparent with gains on 1p and 20q and losses on 9p (Table I).

Discussion

Herein, we present our CGH study on 30 WTs that displayed numerous chromosomal alterations. In regard to only one available CGH study of 15 WTs, previous reported chromosomal aberrations but also novel regions of interest in this tumor have been observed.

Giefing *et al* (17) revealed in his subset of 15 WTs, that losses of chromosome 22, 17p and 12q (73, 53 and 47%, respectively) were the most consistent alterations. Chromosomal gains were observed most frequently on 13q, 4q, 6q and 2q (67, 60, 33 and 27%, respectively). The present study confirms these

commonly observed losses and gains; however, at an approximately one-third lower incidence of the affected tumors when compared to Giefing *et al* (17).

Deletions of the terminal region of 9q were observed in 13% of our tumors and were also noted by Giefing *et al* (17) in their smaller cohort at a percentage of 13% in their tumors. In this region, *GAS1* on 9q21.33 and *TSC1* on 9q34.13 are located. The tumor-suppressor gene *GAS1* has previously been shown to play a role in other entities such as myeloid malignancies (19). Loss of *TSC* genes has been reported to result in tumor development by its constitutive activation of MTOR and downstream signaling elements (20).

In accordance to Giefing *et al* (17), our study revealed additional deletions affecting chromosomes 16, 17 and 22 with the minimal overlapping region on 16p12p13.1, 17p13 and 22q12. Close to the minimal overlapping region on 16p12p13.1 (Fig. 2), several significant candidate genes are located which are known to be involved in cell cycle regulation and apoptosis, for example *MAPK3*, *LITAF* and *ERCC4*. Furthermore, for other tumors such as breast carcinomas, colorectal tumors and anaplastic thyroid cancers, the important role of deletions of the short arm of chromosome 16 has been previously demonstrated (21-23).

Notably, Giefing *et al* (17) as well as our study revealed gains on 6q as a frequent event in WTs. In the delineated consensus region on 6q21, which was affected in 33% of our tumors, the *FYN* oncogene is located. *FYN* belongs to the Src kinases, which are key upstream mediators of both the PI3-K and MAPK signaling pathways, and have been shown to play important roles in cell proliferation, migration and survival (24). In contrast, cytogenetic examination of the cultured tumor cells showed normal karyotypes in the majority of WTs. Nevertheless, one study also revealed non-clonal deletions of chromosome 6 as well as a deletion of 6q21 in 3 of 13 cultured tumors (10). Notable, in malignant salivary gland tumors, i.e. adenoid cystic carcinomas and mucoepidermoid carcinomas, deletions or rare translocations involving the terminal region of the long arm of chromosome 6, were found to be the most consistent alterations (25-28). In addition, certain types of solid tumors, and several types of leukemias and lymphomas are characterized by various deletions of 6q (29).

As novel findings, the present study detected frequent alterations on 8p (30%) and 9p (23%) in this tumor entity. Alterations on 8p may indicate genes involved in DNA damage response and tumorigenesis such as tumor-suppressor genes *MCPH1*, *ANGPT2* and *PINX1* at 8p23.1pter. *CDKN2A* and *CDKN2B*, located at 9p21, are also important candidate genes known to be involved in cell cycle regulation (Fig. 2). As these observations were not detected in previous conventional karyotyping analyses (6,8,10) or comparative genomic hybridization analysis (17), they warrant particular interest in further studies.

In addition to the deletions on 22q12, gains of chromosome 22 were also a frequent event in our WT cohort. The potential candidate tumor-suppressor genes on 22q12 *CHEK2* and *TIMP3* were previously shown to be associated with increased risk of prostate cancer (30) and pancreatic endocrine tumors (31). Moreover, the growth factor PDGFB was found to play an essential role in the regulation of cell proliferation, cell migration and survival (32). Due to the identification of specific

gains at 22q12.3qter, further analysis of PDGFB in WTs is warranted.

Furthermore, conventional karyotyping of WTs revealed a translocation t(11;19)(q21;p13.1) (5,8,9,11). This translocation results in the fusion gene *MAML2/CRTC1*, which is common in most cases of mucoepidermoid carcinoma (MEC) and possibly indicates the derivation of certain MECs from WTs (11,15). Since balanced translocations are not detectable with CGH, no conclusions are possible concerning presentation of the t(11;19) translocation in our cohort.

Notably, a study analyzing a cohort of 15 primary MECs by CGH analysis revealed losses on 15q in 4 of their tumors, partly together with deletions on 8p and 22 (33). Furthermore, a microarray analysis of a salivary duct carcinoma arising in WT also revealed losses of 8, 15 and 22 in addition to other alterations (34). We also observed this pattern of alterations in our WTs, particularly in those with a high number of chromosomal alterations possibly indicating chromosomal instability. Notably, in a variety of other tumor entities such as MEC, head and neck squamous cell carcinoma and prostate cancer, defined copy number alterations, e.g. loss of 8p, as well as the total number of imbalances are of general importance in the progression and also the metastatic potential of these tumors (28,35). Collectively, all these findings support a potential role of this pattern of alterations for a more pronounced tumor type for WT. As none of the patients in this series presented with a tumor recurrence to date, we cannot address this issue in the present study. Nevertheless, our findings need to be interpreted with caution reflecting on the morphological heterogeneity in WTs. In the selected study design, it was not considered to which extent lymphoid or epithelial tumor components were analyzed. Therefore, the identification of different chromosomal aberrations may reflect the different tumor components. However, in defining significant candidate genes based on the CGH findings, one should be aware that chromosomal alterations can point to important genes in tumorigenesis, but may also be the result of chromosomal instability.

To the best of our knowledge, the present study presents the largest cohort of 30 WTs analyzed by CGH to date. Our molecular cytogenetic analysis confirmed the findings of previous cytogenetic studies and identified new recurrent alterations as well as different patterns of chromosomal aberrations with the potential for a diagnostic impact. The presented data identified significant consensus regions that may harbor candidate genes of importance in the tumor biology of WTs. These warrant further study to assess their possible involvement in WT tumorigenesis.

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