SPANDIDOS PUBLICATIONS

Chromosomal imbalances, 11q21 rearrangement and MECT1-MAML2 fusion transcript in mucoepidermoid carcinomas of the salivary gland

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Abstract. The aim of this study was to determine genetic alterations in mucoepidermoid carcinomas of the salivary gland in association with clinical and histopathological parameters. Nineteen formalin-fixed, paraffin-embedded tumors were analysed by using comparative genomic hybridization (CGH), fluorescence *in situ* hybridization (FISH) on interphase nuclei and reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of *MECT1-MAML2* fusion transcript. The CGH analysis showed an overrepresentation of chromosome X and losses of entire chromosomes or regions on chromosome 1, 2, and 15 as the most frequent copy number changes. In 37% of the analysed tumors a *MAML2*-rearrangement by interphase FISH was detected, whereas 58% of the samples showed expression of *MECT1-MAML2* fusion transcript. We conclude that the presence of MAML2-rearrangement as well as of *MECT1-MAML2* fusion transcript may reflect a more favourable prognosis and may be a useful marker for clinical prediction of the biological behavior of these tumors as previously reported.

Introduction

Mucoepidermoid carcinomas (MECs) are rare malignant neoplasms of variable histopathologic differentiation with unpredictable clinical behavior. These tumors showed a wide age distribution with an incidence peak at about the fifth decade of life. MECs are composed of three different cells types: intermediate, epidermoid (squamous) and mucus-secreting cells (1). Approximately half of the tumors occur in the parotid glands. Patients with a high grade carcinoma have an unfavorable outcome; however, the clinical and prognostic impacts of molecular aberrations remain unknown, due to the limited number of reported cases in the literature. Genetic analyses on MECs like G-banding, FISH, SKY and CGH revealed genetic losses at chromosome 9p21, 8p, 5p, 16q and 12p and gains of 7 (2-7).

A specific translocation t(11;19)(q21;p13) is known, which is associated with two types of salivary gland tumors, namely MECs as well as Warthin's tumors (8,9). The Warthin's tumor, a likewise frequently more occurring benign tumor of the salivary gland with distinctive histomorphological features from that of MEC showed the same translocation t(11;19)(q21;p13), which is seen also in 60% of the MECs (10). Further translocations with alternative translocation partner such as t(11;17)(q22;p11) or t(11;13)(24q;q12) could be found in these tumors (11-16). The target genes in the t(11;19)(q21;p13) translocation are known (17). Molecular analysis of the translocation t(11;19)(q21;p13) identified a fusion transcript of the exon 1 of the mucoepidermoid carcinoma translocated-1 gene (*MECT1*, alias *CRTC1*, *TORC1*, *WAMTP1*) at 19p13 with the exons 2-5 of a novel member of the mastermind-like gene family (*MAML2*) at 11q21. The fusion transcript activates the transcription of the Notch target genes such as HES1 and HES5 (18,19). This fusion gene is also shared in lung mucoepidermoid carcinoma (20,21). Clear cell hidradenoma of the skin as the third tumor type with an identical *MECT1-MAML2* gene fusion was reported by Winnes *et al* (22) and Behboudi *et al* (23).

Materials and methods

Formalin-fixed, paraffin-embedded tumor samples. Nineteen tumor samples from 18 patients, diagnosed from 1988 to 2002 were retrieved from the archives of the Institute of Pathology Innsbruck, Austria and of the Institute of Pathology, Salzburg, Austria. Immunohistochemical examination was done with

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MIB-1 antibody (Dako, Austria, dilution 1:100, autoclave 1 bar, in citrate buffer for 30 min) using an automated immunostainer (Nexes, Ventana, Tuscon, AZ, USA). The evaluation of MIB-1 expression was determined as the percentage <10% and >10% of stained cells.

Comparative genomic hybridization (CGH). To evaluate if tumors have other abnormalities except 11q-aberrations, genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor material using standard protocols. Control DNA was prepared similarly from peripheral blood specimens of healthy individuals. Tumor (1 μg) and control (1 μg) DNAs were labeled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively by nick translation (Roche Diagnostics, Mannheim, Germany). After co-precipitation with 40 μg human Cot-1 DNA (Roche Diagnostics) and pre-annealing to suppress signals from repeated sequences the hybridization was carried out to normal human metaphase cells for 3 days at 37˚C. For detection the slides were stained with avidin-fluorescein isothiocyanate (Vector Labs Burlingame, CA) and anti-digoxigenin-rhodamine (Roche Diagnostics). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole and specimens were mounted in antifade solution (Vectashield, Vector Laboratories). Image acquisition was carried out using a fluorescence microscope (Zeiss Axioplan) equipped with a CCD camera (JAI M300) and ISIS software (Metasystems, Altlussheim, Germany). Gains or losses were calculated as significant by the evaluation software when fluorescence ratio values were <0.8 and >1.25. Pericentromeric, heterochromatic, telomeric regions and chromosome Y were excluded from the evaluation.

Identification of t(11;19) by fluorescence in situ hybridization (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of MECT1-MAML2 fusion transcript. FISH analysis. Sections (2 μm) were made from paraffin-embedded tissue blocks. The slide and probe preparations were performed according to the protocol for paraffin specimens of the manufacturer (Vysis Downers Grove, IL, USA) with minor modifications. Briefly, the slides were deparaffinized with three 10-min xylene washes, dehydrated in two 5-min washes in 100% ethanol. Subsequently, the slides were incubated in 10 mM citric acid buffer for 60 min at 80˚C, followed by an incubation in a pepsin solution (0.5 mg/ml), fixed in 4% formaldehyde for 10 min and dehydrated in a ethanol series (70, 90, 100%). To evaluate the CGH results, probes for centromere 2, 7, 16, 17, X, Y, LSI probes for 8q24, 9p21, 13q14, 20q13 and 22q11.2 as well as telomeric probes for 19p and 19q (Vysis) were applied onto the slides in the area of interest. Also a dual break apart probe (ZytoLight MEC I, ZytoVysion, Germany), a mixture of two clone contigs hybridizing to the chromosomal band 11q21 was used. The green-labeled probe (size ~550 kb) hybridizes proximal the *MAML2* gene, the orange-labeled probe (size ~400 kb) distal to *MAML2*.

The slides were covered with a glass cover slip, sealed with rubber cement, placed in the HYBrite system (Vysis) and denatured at 80˚C for 5 min and hybridized overnight at 37˚C. After two wash steps the slides were counterstained with DAPI in antifade solution. At least 165 nuclei (range 165-321, mean 216) were scored in each case with a fluorescence microscope, equipped with specific filters for SpectrumOrange, SpectrumGreen and DAPI. The images were acquired with a CCD camera and ISIS software.

RT-PCR analysis. Total RNA was extracted from 5-10 formalin-fixed, paraffin-embedded tissue sections (5 μm) from 19 MECs using High Pure RNA Paraffin Kit (Roche Diagnostics). Primers for RT-PCR for amplify the MECT1-MAML2 fragment with an expected size of 105 bp were *METC1* 5'-GCCTTCGAGGAGGTCATGA-3' and *MAML2* 5'-CTTGCTGTTGGCAGGAGA-3'. RT-PCR was run using a denaturation step at 94˚C for 3 min, followed by 35 cycles of 94˚C for 45 sec, 55˚C for 30 sec and 72˚C for 30 sec. A final extension step was done at 72˚C for 10 min. To check the quality of the cDNA it was also amplified in a GAPDH fragment with an expected size of 184 bp with the primers forward 5'-TCCATGACAACTTTGGTATC-3' and reverse 5'-TTCAGCTCAGGGATGACCTT-3'.

Results

Clinical and pathological summary. Seventeen samples were primary tumors and two were lymph node metastases. One of these lymph node metastases occurred in a patient with submandibular MEC within the first year after the initial surgical treatment (cases 9a and 9b, Table I). Distant metastases were not documented. Thirteen of the MECs originated from the major salivary glands with the majority located in the parotid gland and 3 cases derived from the submandibular gland. The remaining 2 tumors occurred in a minor salivary gland (soft palate). Tissue samples were stained with haematoxylin and eosin and classified by a pathologist according to the WHO classification (24). The age range at the time of diagnosis was 27-85 years with a median age of 59.4 years. Twelve of the 18 patients (66.7%) were >50 years of age. The male to female ratio was 1:1.6.

All patients presented one or more signs of symptoms. Clinical presentations were relatively uniform. The first symptom in all cases was non-inflammatory, painless solid tumefaction in the area of a salivary gland. Three parotid tumefactions were associated with facial palsy. Two patients experienced discomfort in the parotid region. Six patients (37.5%) had clinically positive cervical lymph nodes at the time of their examination; in all cases cervical metastases were confirmed by neck dissection. No patient had any signs of distant metastases at the time of diagnosis.

The tumors were clinically staged according to the TNM system (24). The sizes of the tumors ranged from 1.5 to 7 cm. Duration of symptoms ranged from 4 weeks to 18 months and did not correlate with tumor site or size. All patients were primarily treated with surgery. For tumors of the parotid gland a superficial (4 cases) or total (9 cases) parotidectomy was performed, supplemented by a suprahomohyoid (12 cases) or radical neck dissection (in 1 case). The 3 MECs of the submandibular gland were treated with suprahomohyoid neck dissection. Surgical management of MECs of minor salivary gland (2 cases) involved the local excision with suprahomohyoid neck dissection. All surgeries

Summary of clinical, histopathological and immunohistochemical data of 19 tumor samples from 18 patients with

Pat. no.	Gender/ Age	Tumor	S	G	TNM	Tumor localization/ tumor size (cm)	MIB-1 Expression	
							<10%	>10%
1	M/31	PT	6	2	pT2N0	Gl. p.r./2.5	+	
2	F/84	PT	3	3	pT3V1N1	Gl. p.r./4.8		+
3	M/42	PT	3	2	pT1N0	Gl. p.r./1.8	+	
4	M/44	PT	3	2	pT2N0	Gl. p.r./3	+	
5	M/73	PT	5	2	pT1N2b	Gl. p.l./2		+
6	M/47	PT	5	1	T1N0	Minor salivary gland /1.5	+	
7	F/65	LNM	5	3	N2bM0	Gl. p./7[a]		+
8	F/60	PT	5	1	T1N0	Gl. p.l./1.5	+	
9a	M/68	PT	3	3	T2N2b	Gl. subm.r./3.5		+
9b		LNM				Gl. subm.		+
10	F/54	PT	5	3	pT3N2b	Gl. p.l./5.5		+
11	F/82	PT	10	3	T4N1	Gl. p./6.5		+
12	F/69	PT	10	1	T2N0M0	Gl. p.l./3	+	
13	F/64	PT	10	3	pT2N0MX	Gl. subm./2.5		+
14	F/32	PT	10	1	T1N0	Gl. p.l./2	NA	
15	F/85	PT	5	3	pT3bN0MX	Gl. p./4.5		+
16	F/27	PT	5	3	pT2N0MXR1	Gl. p.r./3.2	+	
17	M/82	PT	5	2	pT2N1	Minor salivary gland/2.5	+	
18	F/61	PT	10	2	T2N0M0	Gl. subm./2.5	+	

Pat. no, patient number; M, male; F, female; PT, primary tumor; LNM, lymph node metastases; S, overall disease survival (years); G, tumor grading (G1, low grade, G2 intermediate, G3 high grade); Gl. p.r and Gl. p.l., Glandula parotis right and Glandula parotis left; Gl. subm., Glandula submandibularis; neck r, neck right; [a]size of the primary tumor; NA, not analysed.

were performed as curative resections, with all cases considered histopathologically to have tumor-free margins. The surgical margins were defined as negative in cases showing a rim of normal tissue of >3 mm around the tumor. Surgical treatment was supplemented with radiotherapy in 7 cases. The interval between surgery and the start of radiation therapy was 12-28 days. The radiotherapy was delivered using Co-60. Doses ranged from 54 to 70 Gy and the duration of therapy ranged from 35 to 42 days.

Follow-up ranged from 4 to 12 years. Of the 18 patients with MECs, 14 (77.8%) were alive without disease, 2 patients died from other causes (11%) and 2 patients died from their disease (11%). Four patients were free of the disease for 3 years; 8 patients for 5 years, 1 patient for more than 5 years. Five patients lived for >10 years and one of them died from another cause. Three patients developed local recurrence 23, 44 and 67 months postoperatively. Each recurrence (case no. 1, 8, 11) appeared in tumors of the parotid gland. The 5-year overall disease specific survival rate of all patients was 78%. Fifty percent of our tumors (9 cases) showed MIB-1 expression >10%. A summary of clinical and histopathological data is given in Table I.

CGH data. Fifteen primary MECs and 2 lymph node metastasis with sufficient DNA were analyzed with CGH. Copy number alterations were found in 13 of the analyzed 17 MECs (76.5%). In total, we detected 35 losses vs. 27 gains (1:1.29) with an average of 1.59 gains and 2.06 losses per tumor. Three of the 17 cases (17.6%) showed one aberration, 5.9% of the cases one (1/17) and 52.9% of the case (9/17) three or more aberrations. As the most frequent deviations gain on X (29.4%), partial or complete losses on chromosome 1, 2 and 15 (23.5%) were detected, followed by gain of 7/7p, losses of 17pq, 19/19p and 20/20p/20q in 17.6% (Table II; Fig. 1).

FISH data. FISH analysis was performed in all 19 tumors. In 7 of 19 tumors (36.8%) a *MAML2*-rearrangement at 11q21 was detected (Table I). As the most frequent, a signal pattern of one orange/green (yellow) signal (representing a normal 11q21 locus and a separate orange and green signal, demonstrating a disrupted 11q21 region) was seen (Fig. 3). This signal pattern suggests a t(11;19) or a variant of it, involving the 11q21 region. The other frequent chromosomal aberrations detected by CGH were confirmed by FISH.

RT-PCR results. The following RT-PCR-analysis showed the presence of a MECT1-MAML2 fusion transcript in 11 cases (57.9%), including the 7 FISH-positive cases (Fig. 2, not all data shown). A summary of our results is given in Table II.

Table II. Summary of CGH, FISH and RT-PCR results.

Pat. no.	CGH		FISH with 11q21 break apart probe	RT-PCR
	Losses	Gains	MAML2-Rearrangement (% aberrant cells)	Presence of MECT1-MAML2 fusion transcript
1	1pter-p32, 6pter-p21, 15q22-qter, 17, 20q, 22	18p, X	Yes (46)	Yes
2	15q22-qter	14	No	Yes
3	No imbalances	X	Yes (23)	Yes
4	No imbalances	X	Yes (57)	Yes
5	8pter-p21.1, 15	X	No	No
6	No imbalances	No imbalances	No	No
7	9q, 16, 17pter-q22, 18q	2q32.1-q34, 4q26-qter, 5q14-q23.3, 8q	No	No
8	No imbalances	No imbalances	Yes (50)	Yes
9a	No imbalances	19p	No	No
9b	5q11-q23, 14q11-q23, 17pter-q23, 18q,19q	X[a], 3q26-qter, 6q22	No	No
10	No imbalances	No imbalances	No	No
11	1pter-p32. 2[a], 6pter-p21, 10q23-qter, 14q22-qter, 15q22-qter, 22	4, 7, 9p, 13q14-q31, 18	No	Yes
12	No imbalances	No imbalances	Yes (84)	Yes
13	3p, 8p	5p, 7p, 20p	No	Yes
14	1[a], 2q[a]	20q	No	No
15	2q, 3p21.1-p12, 4[a], 13	19	No	Yes
16	1p31.1-p22.1, 2[a]	7, 19, 20	Yes (66)	Yes
17	NA	NA	No	No
18	NA	NA	Yes (56)	Yes

Pat. no, number of patients; [a]imbalances with a clear shift, but the CGH profile shows only a partially significant imbalance (partially reaching the threshold); NA, not analysed.

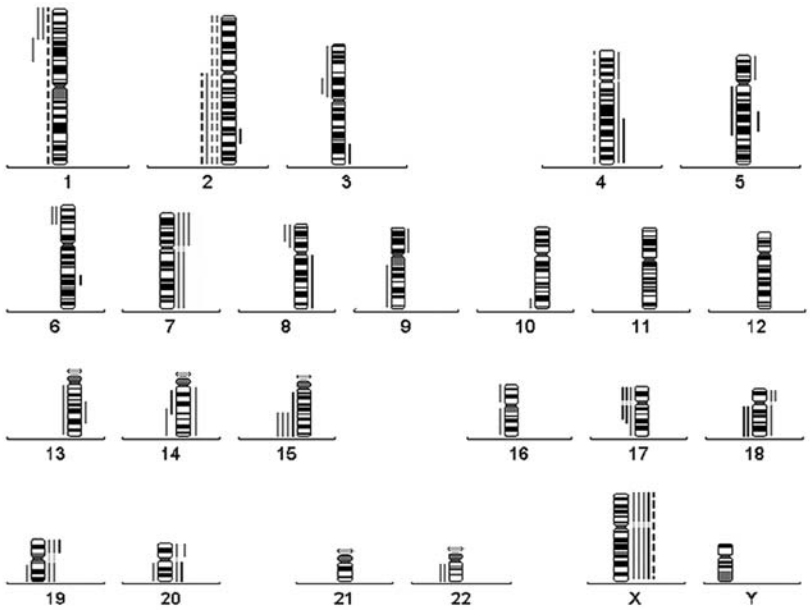

Figure 1. CGH results as summary profile for 17 analyzed MECs. Lines to the left side of the ideograms represent losses, lines to the right side chromosomal gains. Dotted lines indicate imbalances not reaching the diagnostic thresholds; gray lines indicate aberrations deriving from MECT1-MALM2 fusion transcript-positive cases and black lines aberrations from fusion transcript-negative cases.

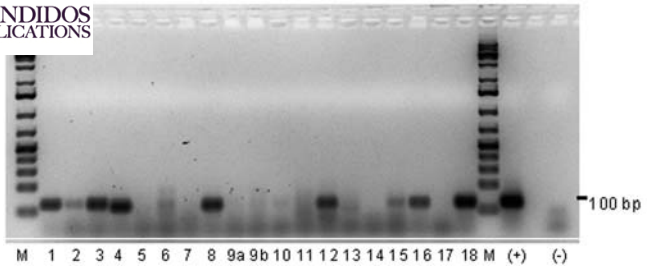

Figure 2. RT-PCR product of MECT1-MAML2 transcript in 19 formalin-fixed, paraffin-embedded tumors of 18 patients (not all data shown). M, marker; (+), positive control; (-), negative control.

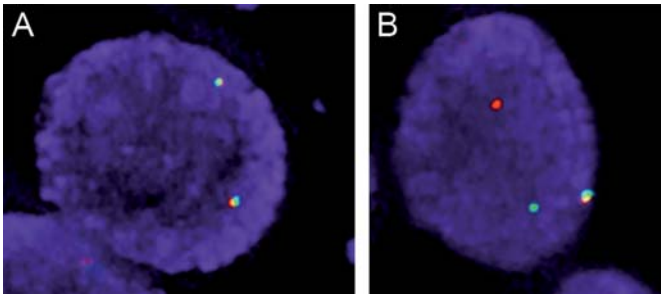

Figure 3. Interphase fluorescence *in situ* hybridization with the dual color, break apart probe MEC I represents MAML2 rearrangement. (A) Nucleus with two orange/green (yellow) signals indicating two normal 11q21 loci (case 2, Table I). (B) Nucleus with one orange/green (yellow) signal and a separate orange and green signal, caused by a break in 11q21 (case 8, Table I). This signal pattern was the most frequent finding in all analyzed cases.

The two small tumor groups with and without 11q-abnormalities did not allow a statistical analysis. Table III shows the association between the clinicopathologic parameters of patients with and without the presence of the 11q21 aberration.

Discussion

The presence of aberrations like translocation t(11;19)(q21;p13) is of special importance for future investigations, providing prognostic and therapeutic relevance. The impact of the METC1-MAML2 fusion on the clinical outcome in MECs is unclear und controversial in the literature. Our data give rise to several commentaries in comparison to the data by other authors:

i) In the present MECs 36.8% of the tumor samples showed 11q21 rearrangement. The RT-PCR analyzed tumors showed a MECT1-MAML2 fusion product in 57.9% of the tumors. This frequency is in agreement with studies on MECs by other authors (11,19). Several authors concluded that fusion positive tumors are biologically less aggressive with better clinicopathological behavior in comparison with fusion transcript negative tumors (11,20,32,33).

Behboudi *et al* (11) demonstrated that patients with fusion gene-positive tumors were substantially younger at clinical presentation, showing a predominance of smaller, high-differentiated low-grade tumors. Fusion-positive patients had a significant lower risk of local recurrence, metastases and tumor-related death. According to the study by Serra *et al* (20) all analyzed pulmonary MECs with 11q21 rearrangement were low-grade tumors. In contrast to these

Table III. 11q21-rearrangement and expression of MECT1-MAML2 fusion transcript in comparison with clinicohistopathological and genetic data.

	MECT1-MAML2-fusion transcript positive (n=11)	MECT1-MAML2-fusion transcript negative (n=7)
Mean age (years) (n=18)	59 (n=11)	60 (n=7)
Mean tumor size (n=18)	3.3 cm (n=11)	3.4 (n=7)
MIB-1 expression (n=18)		
<10%	7 (64%)	2 (29%)
>10%	4 (36%)	5 (71%)
Lymph node metastasis (n=18)		
Present	2 (18%)	5 (71%)
Absent	9 (82%)	2 (29%)
Tumor grade (n=18)		
G I, GII	6	4
G III	5	3
Mean survival time (years) (n=18)	6.36 (n=11)	5.43 (n=7)
No. aberrant CGH-cases	(n=8/10, 80%)	(n=5/7, 714%)
Average gains/aberrant case	2.0	2.2
Average losses/aberrant case	3.25	1.8
Aberrant cases with >2 CNV	5 (62.5%)	4 (80%)

authors in the cohort of Tirado and co-workers (33) the fusion transcript *METC1-MAML2* showed no association with tumor grade. The lacking of the fusion transcript was significantly associated with metastasis, suggesting that fusion-negative tumors represent a group of biologically aggressive tumors.

However, our study size is too small for a meaningful statistical consideration, but our fusion-negative tumors are predominately associated with occurrence of regional metastases (71% vs. 18%). In comparison to the study of Behboudi *et al* (11) the estimated median survival for fusion-positive patients was greater than in fusion-negative patients (10 years vs. 1.6 years). The mean survival time in patients with fusion-gene positive tumors in our collective was higher than in patients with fusions-negative tumors (6.36 years vs. 5.43 years). Regarding patient age, tumor grading and tumor size no difference could be observed between the fusion-negative and fusion-positive tumors.

ii) MIB-1 expression >10% we found predominately (87.5%) in high grade tumors (G3), whereas the most (89%) of the low to intermediate tumors (G1 to G2) were associated with MIB-1 expression <10%. Published immunohistochemical studies demonstrated also that high Ki-67 expression is significantly correlated with higher grade tumors (11,34,35). Regarding the fusion status we found more frequent MIB-1 expression <10% in fusion transcript-positive tumors than in MECT1-MAML2-negative tumors (77.8 vs. 22.2%). The part of our samples with expression greater than 10% was slightly lower in fusion-positive tumors (44.4 vs. 55.6%). In addition to our findings, the status of Ki-67-expression supplies additional prognostic information regarding tumor behavior.

iii) Extensive and systematic molecular genetic data for malignant tumors of the salivary gland have not been previously reported. Previous cytogenetic studies (by G-banding, SKY, FISH) of MECs of the salivary gland reported other, besides the common t(11;19), aberrations like -Y, +5, +7, +8, -14, +X and translocations involving chromosome 1, 3, 5, 6, 8, 12, 13, 15, 16 and 20 (11,15,21,25-30). To our knowledge, only two MEC cell lines with CGH data are available in the literature (21). In the first cell line gains or amplifications on 1q31, 5p, 6p22, 7pter-p15, 8q21.3-qter, 11p13-qter, 15q25-qter and losses of chromosome 9 and 20 were found. Frequent aberrations in the second analyzed cell line were gains at 3pter-p24, 3q, 7pter-q11.2 and 20. The regions 3p21-p13, 4p16, 4q32-qter, 5q32-qter and 8pter-p12 were under-represented.

The most frequent findings in our tumors were gains of X in 5 cases, partial or complete losses on chromosome 1, 2 and 15 in 4 cases, followed by gain of 7/7p, 19/19p and 20/20p/20q. MECs are characterized by a t(11;19)(q21;p13) translocation, more often occurring as sole anomaly being a simple translocation or complex one (6,31). The findings are only in partial concordance with the data by Tonon *et al* (21) and Behboudi *et al* (23) regarding gain on chromosome 7, 20, X and loss of 8p and 15.

iv) Copy number variation (CNV) and presence of MCET1-MAML2 fusion transcript: patients with fusion gene-positive tumors showed higher CNV (5.25 vs. 4.0) in fusion-negative tumors; whereas MECT1-MAML2-positive tumors showed more losses than gains/case (3.25 vs. 2.0) the number of losses and gains/case was approximately equal (1.8 losses vs. 2.2 gains) in fusion transcript negative tumors. In 62.5% of the fusion-positive tumors we detected more than 2 CNV in comparison with 80% in the fusion-negative tumors. Regarding the CGH-aberration spectrum, losses of 6p, 8p, 22 and gain of chromosome 7 and 18 were found only in MECT1-MAML2-positive tumors.

We concluded that the presence of MECT1-MAMl2 fusion transcript in MECs may define, in view to clinical and pathological outcome, a subset of tumors with more favorable outcome. Our findings in MECT1-MAML2-transcipt-positive tumors as a subgroup of MECs with regard to a better clinicopathological outcome is in agreement with most other publications. It remains to be clarified, if the presence of this aberration represents a useful diagnostic marker for prognosis and prediction of the biological tumor behavior.

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